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Breast cancer specific gene 1 (BCSG1) is not expressed in normal breast tissue but is highly expressed in the vast majority of invasive and metastatic breast carcinomas. When over expressed, BCSG1 significantly stimulates the proliferation and invasion of breast cancer cells. However, the mechanisms that turn on the BCSG1 transcription in breast cancer cells were largely unknown. In the first year of funding, we extensively examined the methylation status of a CpG island located in the exon 1 of BCSG1 gene in a panel of breast tumor-derived cell lines to determine whether DNA methylation plays a crucial role in BCSG1 expression. *In vivo* bisulfite DNA sequencing of genomic DNA isolated from breast cancer cell lines showed that the 15 CpG sites within the CpG island were completely unmethylated in all BCSG1-positive cell lines (5/5), but were densely and heterogeneously methylated in the majority of BCSG1-negative cell lines (3/4). A correlation between hypomethylation of the exon 1 and expression of BCSG1 mRNA was also verified in primary breast tumor tissues. In addition to the methylation control we also demonstrate that the binding of c-jun to two Ap1 sites located in the first intron is critical for BCSG1 transcription in cells that have unmethylated BCSG1 gene. Inhibition of Ap1 transactivation by using its dominant negative mutant severely reduced BCSG1 expression in breast cancer cells.

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INTRODUCTION

Previous studies conducted by differential DNA sequencing and *in situ* hybridization have identified BCSG1 (1), also referred as synuclein γ (2) or persyn (3), as a breast cancer specific gene due to its distinct expression pattern in breast tissues. BCSG1 is not expressed in normal breast tissue or tissues with benign breast diseases, but is highly expressed in the vast majority of stage III/VI breast carcinomas (1;4). BCSG1 expression in advanced breast carcinomas is not merely adventitious, but plays a positive role in the process of invasion and metastasis. It has been demonstrated that exogenous expression of BCSG1 in breast cancer cells led to a significant increase in cell motility and cell proliferation in cell culture (5;6), and a profound augmentation of metastasis in nude mice (5). Thus far, BCSG1 gene mutations or amplifications have not been found by examination of breast carcinoma specimens and breast cancer cell lines. Therefore, it was suggested that the abundant expression of BCSG1 protein and mRNA in breast carcinomas is not caused by mutation or gene amplification but by transcriptional activation (3;4).

To elucidate the molecular and cellular mechanisms that control BCSG1 transcription in breast cancer cells, we previously isolated a 2.2 kb fragment of human BCSG1 gene that includes 1 kb of the 5'-flanking region, exon 1, and intron 1 (7). Sequence analysis indicates that the exon 1 region contains a CpG island. In *vivo* genomic bisulfite sequencing demonstrates that the BCSG1 CpG island is totally unmethylated in BCSG1-positive SKBR-3 and T47D cells but partially methylated in BCSG1-negative MCF-7 and HepG2 cells (7). This suggests that DNA demethylation may be an important factor contributing to the aberrant expression of BCSG1 in breast cancer cells. However, treatment of MCF-7 or HepG2 cells with a specific methyltransferase inhibitor 5-Aza-2'-deoxycytidine that demethylated the CpG island of BCSG1 only induced low levels of BCSG1 mRNA in these cells, suggesting that there are DNA methylation-independent mechanisms responsible for the abundant expression of BCSG1 in T47D and SKBR3 cells. It is possible that BCSG1 expression is controlled by DNA methylation-dependent and independent mechanisms. Demethylation of cytosine of the CpG dinucleotide motifs within exon 1 may allow initiation of gene transcription, upon release of the transcriptional silencing, BCSG1 transcription is further activated by transcription factors that interact with the cis-acting elements residing in the regulatory regions of the BCSG1 gene.

Therefore, during the first year of funding, we extensively investigated the possible role of DNA methylation as the primary determination factor in BCSG1 expression and the functional role of the transactivator Ap1 in the activation of BCSG1 expression in breast cancer cells.

BODY

1. Hypomethylation of the BCSG1 gene CpG island promotes its aberrant expression in breast carcinoma (appendix I)

To fully understand the molecular mechanisms underlying the abnormal expression of BCSG1 in neoplastic diseases, in this study, we extensively examined the methylation status of a CpG island located in exon 1 of SNCG gene in a panel of breast tumor-derived cell lines to determine whether DNA methylation plays a crucial role in BCSG1 expression. *In vivo* bisulfite DNA sequencing of genomic DNA isolated from breast cancer cell lines showed that the 15 CpG sites within the CpG island were completely unmethylated in all BCSG1-positive cell lines (5/5), but were densely and heterogeneously methylated in the majority of BCSG1-negative cell lines (3/4). The methylation occurred primarily at the CpG sites 2, 5, 7, and 10-15. A correlation between hypomethylation of the exon 1 and expression of SNCG mRNA was also observed in primary breast tumor tissues. The importance of DNA methylation in the control of BCSG1 expression in cancer cells is further strengthened by demonstration of re-expression of BCSG1 mRNA in BCSG1-negative breast cancer cells with a demethylating agent 5-Aza-2'-Deoxycytidine. In addition, we demonstrate that inhibition of cell growth leads to a decreased mRNA expression and an increased DNA methylation of BCSG1 gene. Taken together, these new findings strongly suggest that DNA hypomethylation is an important mechanism underlying the abnormal expression of this candidate oncogene in breast carcinomas.

2. Blockade of AP1 transactivation abrogated the abnormal expression of the breast cancer specific gene 1 in breast cancer cells (Appendix II)

Accumulated evidence suggests that the aberrant expression of BCSG1 in breast carcinomas is caused by transcriptional activation of the BCSG1 gene. However, the transcription factors that activate BCSG1 transcription have not been identified. In this study, we extensively investigated the role of AP1 in BCSG1 expression in breast cancer cells. We demonstrate that there are 2 closely located AP1 binding sites residing in the first intron of the BCSG1 gene. Mutation of either AP1 motif on the BCSG1 promoter constructs markedly reduces the promoter activity. We further show that TPA increases BCSG1 mRNA expression and upregulates BCSG1 promoter activity through the intronic AP1 sites. The effect of TPA on BCSG1 transcription is also demonstrated under *in vivo* conditions in intact cells by using chromatin immunoprecipitation assays that show the TPA-induced binding of c-jun to the chromatin region encompassing the intronic AP1 sites. Finally, to examine the direct effect of AP1 transactivation on BCSG1 expression, we established stable cell lines of T47D that express the dominant negative mutant of c-jun, TAM67. RT-PCR and western blot analyses demonstrate that levels of BCSG1 mRNA and protein in TAM67 transfectants were drastically reduced as compared to mock-transfected cells. Furthermore, inhibition of BCSG1 expression by blocking AP1 transactivation produced a similar repressive effect on cell growth as that by expressing BCSG1 antisense mRNA. We show that the anchorage-independent growth of T47D cells expressing either TAM67 or BCSG1 antisense mRNA was significantly inhibited. Taken together, we provide strong evidence to demonstrate that AP1 plays an overriding role in the transcription of the BCSG1 gene and that blockade of AP1 transactivation down regulates BCSG1 expression and suppresses tumor phenotype.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a stable cell line (T47D-TAM67) that express the dominant negative mutant of c-jun
- Establishment of a stable cell line (T47D-BCSG1-As) that express the antisense mRNA of BCSG1
- Establishment of the method to rapidly determine the methylation status of BCSG1 gene

REPORTABLE OUTCOMES

- Two manuscripts have been published in the first year of the award.

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Hypomethylation of the *Synuclein* γ Gene CpG Island Promotes Its Aberrant Expression in Breast Carcinoma and Ovarian Carcinoma[1]

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ABSTRACT

Recent studies indicate that *synuclein γ* (*SNCG*) gene, located in chromosome 10, participates in the pathogenesis of the breast and ovary. SNCG, also known as *breast cancer-specific gene 1* (*BCSG1*), is not expressed in normal mammary or ovarian surface epithelial cells but is highly expressed in the vast majority of advanced staged breast and ovarian carcinomas. When overexpressed, *SNCG* significantly stimulates breast cancer proliferation and metastasis. To fully understand the molecular mechanisms underlying the abnormal expression of SNCG in neoplastic diseases, in this study, we extensively examined the methylation status of a CpG island located in exon 1 of *SNCG* gene in a panel of breast and ovarian tumor-derived cell lines to determine whether DNA methylation plays a crucial role in SNCG expression. *In vivo* bisulfite DNA sequencing of genomic DNA isolated from breast cancer cell lines showed that the 15 CpG sites within the CpG island were completely unmethylated in all SNCG-positive cell lines (5 of 5), but were densely and heterogeneously methylated in the majority of SNCG-negative cell lines (3 of 4). The methylation occurred primarily at the CpG sites 2, 5, 7, and 10–15. Similarly, we observed a strong correlation of hypomethylation of the CpG island and SNCG expression in ovarian cancer cell lines (5 of 5). Intriguingly, the methylation pattern in ovarian cancer cells is different from that in breast cancer cells. In SNCG-nonexpressing ovarian cancer cells, all 15 of the CpG sites were completely methylated instead of selective methylation at certain sites shown in breast cancer cells, thereby suggesting a tissue-specific methylation pattern. A correlation between hypomethylation of the exon 1 and expression of SNCG mRNA was also observed in primary breast tumor tissues. The importance of DNA methylation in the control of SNCG expression in cancer cells is further strengthened by demonstration of re-expression of SNCG mRNA in SNCG-negative ovarian and breast cancer cells with a demethylating agent 5-aza-2′-deoxycytidine. In addition, we demonstrate that inhibition of cell growth leads to a decreased mRNA expression and an increased DNA methylation of *SNCG* gene. Taken together, these new findings strongly suggest that DNA hypomethylation is a common mechanism underlying the abnormal expression of this candidate oncogene in breast and ovarian carcinomas.

INTRODUCTION

Recently, methylation of DNA at CpG dinucleotides has been recognized as an important mechanism for regulation of gene expression in mammalian cells. Methylation of cytosines in the CpG sequence located in the promoter region or exon 1 is thought to ensure the silencing of certain tissue-specific genes in nonexpressing cells. Aberrant methylation is now considered an important epigenetic alteration occurring in human cancer. Hypermethylation of normally unmethylated tumor suppressor genes correlates with a loss of expression in cancer cell lines and primary tumors (1–4). On the other hand, failure to repress genes appropriately by abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes could result in the loss of tissue specificity and could promote cancer formation (5, 6). Numerous investigations suggest that hypermethylation of promoter CpG islands correlates with transcriptional inhibition in neoplasmas. Most of the hypermethylated CpG islands are located in the promoter region of tumor suppressor genes or DNA repair genes, and DNA methylation is associated with the loss of gene expression in cancer cell lines and primary tumors. However, compared with an extensive list of tumor suppressor genes or cell cycle-regulated genes that are silenced in cancer cells to date, only a few genes have been shown transcriptionally reactivated by DNA demethylation in cancer (7). Although the global hypomethylation in cancers has been observed for several years, it has not received much attention until recently. Initially, hypomethylation of human growth hormone, *α-globin*, and *γ-globin* in cancers was observed by Feinberg and Vogelstein (8). These genes are methylated in normal tissues and become hypomethylated in cancers. Later on, the study was extended to various grades of tumors like benign and malignant colon neoplasmas, and hypomethylation was observed in both types of tumors (9). The hypomethylation and overexpression of proto-oncogenes c-*Myc* and c-*Jun* has been detected in chemically induced tumors in mouse liver (10). Rosty *et al.* (11) has recently reported hypomethylation of *S100A4* gene in pancreatic cell carcinomas. A correlation of hypomethylation and expression of MN/CA9 (a tumor-associated antigen) was reported by Cho *et al.* (12) in renal cell carcinomas. Additionally, DNA hypomethylation and overexpression has been shown for *MDR* in myeloid leukemias (13), *BCL-2* in chronic lymphocytic leukemias (14), *MAGE-1* in melanomas (15), and c-Ha-RAS in gastric carcinomas (10). Recently, Strichman-Almashanu *et al.* (16) have identified unique CpG islands that are methylated specifically in normal tissues and not in cancers.

SNCG,[3] also referred to as the *BCSG1* (*breast cancer specific gene 1*), is a member of a neuronal protein family synuclein and its expression is highly tissue specific (17–21). The SNCG protein is abundantly expressed in the peripheral nervous system such as primary sensory neurons, sympathetic neurons, and motor neurons. However, this tissue specificity was apparently lost during the disease progression of breast cancer and ovarian cancer, because this gene, normally silent in breast tissue and ovary, became abundantly expressed in the vast majority of the advanced staged breast carcinoma and ovarian carcinoma.

The involvement of SNCG in human neoplastic diseases first came to light 5 years ago when SNCG was isolated from a human breast tumor cDNA library and was shown to be overly expressed in infiltrating ductal carcinomas (22). By using *in situ* hybridization, Jia *et al.* demonstrated a stage-specific expression pattern of SNCG mRNA, varying from virtually no detectable expression in normal or benign

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[2] To whom requests for reprints should be addressed, at (154P), VA Palo Alto Health Care System, 3801 Miranda Avenue, Palo Alto, CA 94304. Phone: (650) 493-5000, extension 64411; Fax: (650) 849-0251; E-mail: Jingwen.Liu@med.va.gov.

[3] The abbreviations used are: SNCG, synuclein γ; 5-Aza-C, 5-aza-2′-deoxycytidine; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMEC, human mammary epithelial cell; MSP, methylation-specific PCR; OM, oncostatin M; RT, reverse transcription.

breast tissues to low level and partial expression in low-grade ductal carcinoma *in situ* (DCIS) to high expression in advanced infiltrating carcinomas. Immunohistochemical studies to examine SNCG protein expression showed a similar pattern in that it was not detected in normal breast tissues but was detected in a high percentage of stage III/IV breast ductal carcinomas (23). SNCG expression in advanced breast carcinomas is not merely adventitious, but plays a positive role in the process of invasion and metastasis. Our groups and others have demonstrated that exogenous expressions of SNCG induced a more aggressive and invasive phenotype in the breast cancer cell line MDA-MB435 (24), and the inhibition of SNCG with SNCG antisense mRNA reversed the malignant phenotypes of T47D cells (25).

The first piece of evidence suggesting a possible role of SNCG in the development of ovarian carcinoma came from an analysis of the expressed sequence tag database. Lavedan *et al.* (20) noticed that 37% of the human SNCG sequences were originated from an ovarian tumor library, and the rest of the sequences were from brain and breast tumor libraries, thereby predicting that SNCG may also be overexpressed in ovarian tumor. This speculation was confirmed later by Bruening *et al.* (23), who conducted a study to examine SNCG expression in normal ovarian tissue samples and tissues from ovarian carcinoma. This study found that ovarian epithelial cells or ovarian stromal cells from normal ovaries were not stained with anti-SNCG antibody. In contrast, Bruening *et al.* showed that 33 (73%) of 45 ovarian carcinomas strongly reacted with anti-SNCG antibody, and the immunoreactivity was exclusively in malignant ovarian epithelial cells.

To elucidate the mechanisms that underlie the abnormal expression of SNCG in breast cancer cells, a 2.2 kb-fragment of human *SNCG* gene including 1 kb of the 5′-flanking region (−1260 to −170), exon 1 (−169 to +121), and intron 1 (+122 to +935), was isolated in the VA Palo Alto laboratory (26). Our previous studies of SNCG promoter activity in two SNCG-positive and one SNCG-negative breast cancer cell lines suggested that SNCG transcription is primarily controlled by regulatory sequences located in intron 1 and exon 1 but not in the 5′ flanking region.

The intron 1 contains two closely located AP1 recognition sequences. Deletion of these motifs greatly diminished the SNCG promoter activity, suggesting that AP1 is an important transactivator for SNCG transcription in breast cancer cells. Sequence analysis identified a CpG island in exon 1 that contains 15 CpG sites, covering the region −169 to +81, relative to the translation start codon. By using the sodium bisulfite DNA sequencing technique that examined the *in vivo* methylation pattern of the exon 1 region, we found that the CpG sites within the CpG island and its vicinity were partially and heterogeneously methylated in SNCG-negative MCF-7 cells but unmethylated in SNCG-positive SKBR-3 and T47D cells (26).

These initial observations suggest that demethylation of SNCG exon 1 could play a causative role for the expression of SNCG in cell culture. To determine whether demethylation of the exon 1 is a common molecular determinant responsible for the abnormal expression of SNCG in breast carcinoma and ovarian carcinoma, in the present study, we extensively examined the methylation status of exon 1 and SNCG expression in a panel of breast cancer and ovarian cancer cell lines, and in primary breast tumor and normal breast tissues.

Materials and Methods

Cells and Culture Conditions. The breast cancer cell lines, AU565, MCF-7, MDA-MB435, MDA-MB231, MDA-MB 468, T47D, SKBR-3, were cultured in RPMI 1640 with 10% FBS. H3922 was grown in Iscove's modified Dulbecco's medium in the presence of 10% FBS and BT-20 was grown in DME H-16 50% and F-12 50% media supplemented with 10% FBS. The normal mammary epithelial-derived cell line MCF10A was obtained from American Type Culture Collection (ATCC) and cultured according to the instruction provided by ATCC. Two normal human primary mammary epithelial cell lines 184 and 048R with finite life span were cultured in 1:1 mixture of DME/F-12 supplemented with 0.5% FBS, 10 μg/ml insulin, 5 ng/ml epidermal growth factor, 0.1 μg/ml hydrocortisone, and 1 ng/ml cholera toxin. The third finite life span primary cell line derived from the organoid 240L was cultured in complete MEGM medium (Clonetics) supplemented with 10^{-5} M isoproterenol. All HMECs and organoids, and the organoid-derived cell line (240L) were kindly provided by Dr. Martha R. Stampfer at the Lawrence Berkeley National Laboratory, in Berkeley, CA.

The ovarian carcinoma-derived cell lines (A2780 and OVCAR-3, -4, -5, -8) were maintained in DMEM supplemented with 10% FBS, glutamine, and insulin (0.2 IU/ml pork insulin; Novagen; Ref. 27). Human ovarian surface epithelial cell lines were derived as described previously (28). Cells were maintained in a 1:1 mixture of Media 199 and MCDB-105 media, supplemented with 4% FBS and 0.2 IU/ml insulin (29). The life spans of the human ovarian surface epithelial cells (HIO-103, -105, -107, -135) have been extended by ectopically expressing SV40 large T-antigen. These cell lines are nontumorigenic with the exception of HIO-118, which has been shown to form tumors in mice.[4]

Isolation of Genomic DNA from Cell Lines and Tissues. The genomic DNA was isolated from various cell lines by using Promega's wizard DNA isolation kit according to the manufacturer's instructions. Primary breast tumor tissues and normal breast tissues were obtained after surgical resection and stored frozen at −80° C. The tissues were incubated at 55°C in homogenization buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Tween 20, and 5 mg/ml proteinase K for 3 h, and then genomic DNA was isolated using Promega's DNA isolation kit. Donors of tissue specimens agreed to allow their specimens to be used for research purposes. Breast tumors were staged following standard American Joint Committee on Carcinoma/International Union Against Carcinoma tumor-node-metastasis (TNM) methodology.

Genomic Bisulfite DNA Sequencing. Two μg of genomic DNA from each sample was modified by sodium bisulfite as described previously (2). The modified DNA was amplified with primer SNCG-S2F and SNCG-S2R covering the region −275 to +140. PCR reactions were performed in a volume of 50 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pM of each primer, and 2.5 units of platinum Taq polymerase (Life Technology Inc.). PCR reaction was carried out at 94°C for 1 min, and 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally 72°C for 5 min. The 415-bp PCR product was gel purified and ligated into PCR2.1 Topo cloning vector (Invitrogen, Carlsbad, CA). After transformation, individual colonies were picked, and the insert was PCR amplified as described above and sequenced using SNCG-S2R as the primer. Table 1 provide the sequences of the oligonucleotide primers used in this study.

RT-PCR Analysis of SNCG mRNA. For cell lines, Ultraspec RNA reagent (Biotecxs Laboratory, Houston, TX) was directly added to the monolayer cell culture grown in culture dishes. For isolation of RNA from tumor tissues, 50–200 mg tissues were homogenized in 2–3 ml of Ultraspec RNA reagent on ice using polytron homogenizer at the setting of 3 with three 10-s bursts. RNA was then isolated from the lysate according to the vendor's protocol. The RT was conducted with random primers (Promega) using Superscript II (Invitrogen). The PCR reaction was carried out at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with initial activation of the enzyme at 94°C for 1 min. Thirty-eight cycles were performed for SNCG and 26 cycles for GAPDH. The PCR was performed using primers SNCG-RT 5′ and SNCG-RT 3′for SNCG and primers GAPDH-RT 5′ and GAPDH-RT 3′ for GAPDH.

Western Blot Analysis. Total cell lysates were isolated from cells as described previously (30). Fifty μg of protein from total cell lysate per sample was separated on 15% SDS PAGE, transferred to nitrocellulose membranes, blotted with goat anti-SNCG polyclonal antibody (E-20, sc-10698; Santa Cruz Biotechnology) at 1:200 dilution using an enhanced chemiluminescence (ECL) detection system (Amersham). Membranes were stripped and reblotted with anti-β-actin monoclonal antibiotic (Sigma) to normalize the amount of protein loaded on gels.

MSP. Two μg of genomic DNA isolated from ovarian cell lines was treated with sodium bisulfite and purified using Promega's DNA clean-up kit.

[4] A. K. Godwin, unpublished observations.

Table 1 *Sequences of SNCG gene-specific primers*

Primer	Nucleotide sequence (5′ to 3′)
RT-PCR primers	
SNCG-RT 5′	CAAGAAGGGCTTCTCCATCGCCAAGG
SNCG-RT 3′	CCTCTTTCTCTTTGGATGCCACACCC
GAPDH-RT 5′	CCATCACTGCCACCCAGAAGAC
GAPDH-RT 3′	GGCAGGTTTTTCTAGACGGCAG
Bisulfite sequencing PCR primers	
SNCG-S2F	GGTTGAGTTAGTAGGAGTTTA
SNCG-S2R	CCTACCATACCCCACTTACCC
MSP primers	
SNCG-U1CF	GGTTTTTGTATTAATATTTTATTGGTG
SNCG-U2R	ACAAAACTAAATCTCCCTACAAACTACAA
SNCG-M1F	TCGTATTAATATTTTATCGGCGT
SNCG-M2R	ACGAAACTAAATCTCCCTACGAACTACGT
SNCG-WF	ACGCAGGGCTGGCTGGGCTCCA
SNCG-WR	CCTGCTTGGTCTTTTCCACC

The treated DNA was dissolved in 20 μl of water, and 2 μl was used for MSP. The primers specific for unmethylated DNA were SNCG-U1CF and SNCG-U2R; the primers for methylated DNA were SNCG-M1F and SNCG-M2R. These two sets of primers were designed to amplify the same region of exon 1 from −139 to −37, covering the CpG sites 2–11 and yielding a product of 102 bp. The PCR conditions for both sets of primers were as follows: first cycle at 94°C for 1 min to activate the hot start enzyme, then 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final elongation at 72°C for 5 min.

5-Aza-C Treatment. The SNCG-negative breast cancer cell lines (MCF-7 and MDA-MB435) and ovarian cell lines (HIO-135, OVCAR4, and OVCAR8) were cultured in medium containing 0, 1, 5, or 10 μM of 5-Aza-C for 6 days. The medium and drug were replaced every 24 h.

Stable Transfection of MCF-7 Cell Line. MCF-7 cells were transfected with pCIneo vector alone (mock transfected) or with pCIneo-SNCG, and the pooled transfectants were selected by adding 300 μg/ml of G418. The expression of SNCG was confirmed by Western blotting and the cells were further used for proliferation assays.

Proliferation Assay. For HMEC-184, 1.2×10^4 cells were seeded in 24-well plate and treated with 50 ng/ml of OM for 1 and 3 days. [^{3}H]thymidine incorporation was done as described previously (31). For mock- and SNCG-transfected MCF-7 cells, 1×10^3 cells were seeded in black 96-well plates in RPMI medium containing 10% FBS and were harvested at indicated intervals of time. The total DNA content was estimated using cyquant cell proliferation assay kit (Molecular Probes) as per the manufacturer's instructions.

Flow Cytometry Analysis. MCF7-SNCG or MCF7-neo cells were seeded at a density of 2×10^5 in 100-mm dishes in RPMI containing 10% serum. After 24 h, the medium was replaced with RPMI having 0.5% serum. The cells were harvested after 48 h, and DNA content was analyzed as described earlier (32).

Statistical Analysis. Comparisons of experimental data were analyzed by a two-tailed Student's *t* test. A $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Examination of SNCG mRNA Expression in Malignant and Normal Mammary Epithelial Cell Lines. Total RNA was isolated from 10 breast cancer cell lines, and RT-PCR was conducted using specific primers for SNCG and a housekeeping gene, *GAPDH*. SNCG mRNA was detected in 6 of 10 cell lines including AU565, BT-20, SKBR-3, T47D, H3922, and MDA-MB231 (Fig. 1*A*, and data not shown). The remaining four cell lines either showed no expression of SNCG mRNA such as MCF-7, H3396, and MDA-MB435 or expressed this gene at a very low level (Fig. 1*A*, *MDA-MB468*). In contrast, RT-PCR detected equal expression of GAPDH mRNA in all of the cell lines. Western blot analysis (Fig. 1*B*), using specific anti-SNCG antibody, demonstrated SNCG protein expression in the same six cell lines that express the mRNA but not in the four cell lines that express no mRNA or a trace amount, thereby providing a solid validation for the results of the RT-PCR.

We further examined SNCG mRNA and protein expression in one normal mammary epithelium-derived cell line, MCF10A, and 2 primary normal HMEC lines, HMEC-184 and HMEC-048R. Unlike MCF10A cells that can grow in culture indefinitely, the HMEC lines have limited life span. Fig. 1*C* shows that MCF10A expressed a trace amount of SNCG mRNA, as detected by RT-PCR, but SNCG protein could not be detected by immunoblotting. Unexpectedly, both of the HMEC cells showed reasonable levels of SNCG mRNA and protein, which contradicted previous studies by *in situ* hybridization and Western blot that neither detected the mRNA or the protein of SNCG using normal human breast tissues, suggesting that culturing *in vitro* may induce *SNCG* gene expression

Methylation Status of SNCG Exon 1 in Malignant and Normal Mammary Epithelial Cell Lines. To identify the correlation between DNA methylation of exon 1 and expression of SNCG, we examined the *in vivo* methylation status of all of the cell lines by genomic bisulfite sequencing. Genomic DNAs were treated with sodium bisulfite, and the modified DNAs were amplified with the primer SNCG-S2F and SNCG-S2R. This primer set specifically amplified the modified sense strand of *SNCG* gene from −275 to +140, covering the entire exon 1 and its vicinity. The results obtained from 12 cell lines are summarized and are shown schematically in Fig. 2.

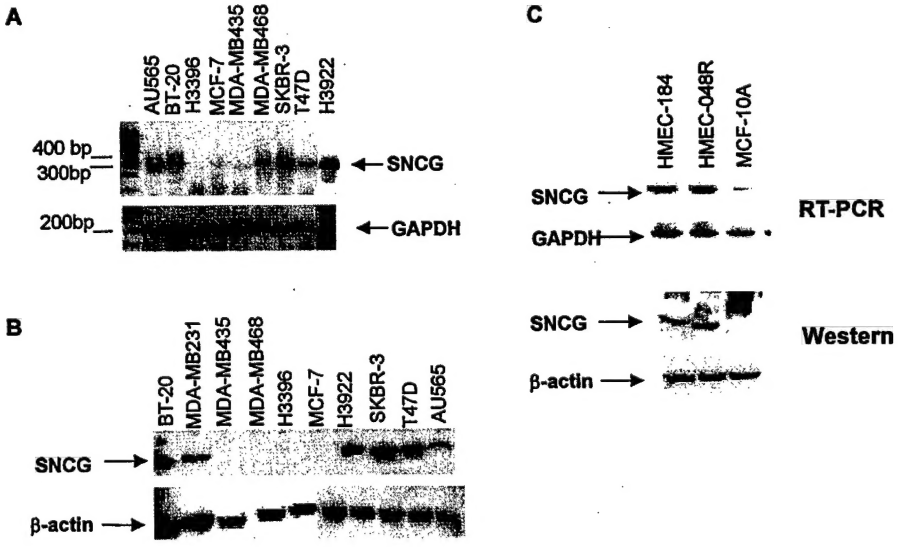

Fig. 1. SNCG expression in breast cancer cell lines and normal HMEC lines. *A*, SNCG mRNA expression in 10 breast cancer cell lines was examined by RT-PCR analysis. One μg of total RNA was used in the reaction of RT in a volume of 20 μl. Two μl of the RT product was used in PCR with specific primers to SNCG or GAPDH. The RT-PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. *B*, SNCG protein expression in the same cancer cell lines was examined by Western blot by using 20 μg of protein of total cell lysate. *C*, SNCG expression in two finite life-span HMECs (184 and 048R) and one immortalized HMEC line (MCF10A) was examined by RT-PCR (*top panel*) and Western blot (*bottom panel*) as described in *A* and *B*.

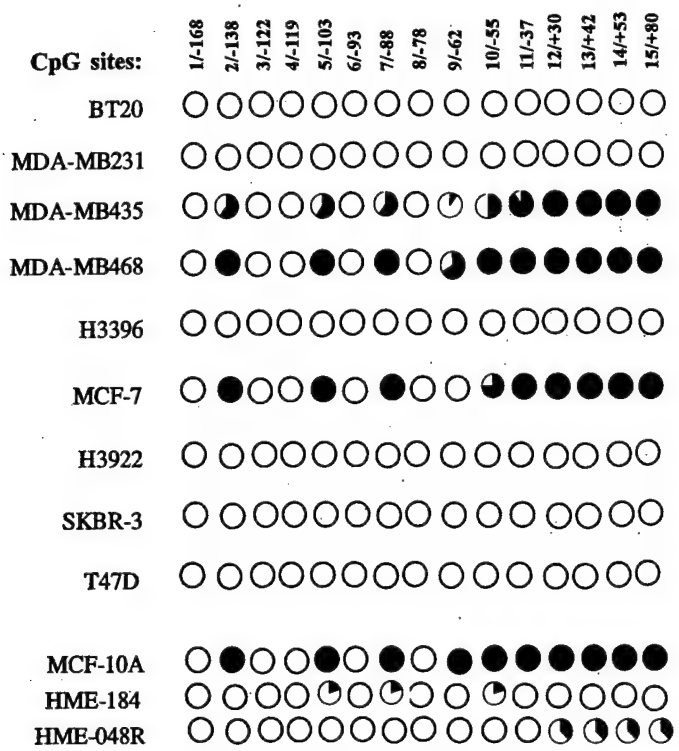

Fig. 2. Methylation status of CpG island in SNCG exon 1 region in various breast cancer cell lines. Genomic DNA was isolated from different cell lines and was modified by sodium bisulfite. Primers SNCG-S2F and SNCG-S2R were used to amplify the region −275 to +140 of modified sense strand of SNCG. CpG positions are indicated relative to the translation start codon, and each circle in the figure represents a single CpG site. For each cell line, the percentage methylation at a single CpG site is calculated from the sequencing results of 6–8 independent clones. ●, 100% methylation; ○, 0% methylation.

The data demonstrated a specific and consistent pattern of methylation in four of five SNCG-negative cell lines. In MCF10A cells, the CpG sites, 2, 5, 7, and 10–15 CpG, were consistently methylated, a pattern identical to the other three cancer cell lines (MDA-MB435, MDA-MB468, and MCF-7). In contrast to SNCG-negative cells, all SNCG-positive cell lines contain unmethylated exon 1. H3396 is the only cell line that has unmethylated CpG island but did not express the *SNCG* gene. In this cell line, repression of transcription by other factors such as lack of transcriptional activator or expression of repressor, may contribute to the loss of SNCG expression despite promoter demethylation. Nevertheless, the results presented in Figs. 1 and 2 clearly demonstrate that the silencing of SNCG expression in mammary epithelial cell lines is correlated predominantly with methylation of exon 1 at specific CpG sites regardless of whether they were originated from breast tumor or from normal breast tissue.

Examination of SNCG mRNA Expression and the Status of Exon 1 Methylation in Primary Breast Tumors. Because the above data confirmed a role of demethylation in *SNCG* gene expression in breast cancer cells in culture, we wanted to know whether the demethylation-dependent gene expression occurs under *in vivo* conditions as well. First, using RT-PCR assays, we examined SNCG mRNA expression in 10 breast tumors, most of which were diagnosed as invasive ductal carcinoma with histological grade of II and above. The pathological characteristics of the tumor samples are listed in Table 2. The expression level of SNCG mRNA in the tumor-adjacent normal tissue from each patient was also determined. In this experiment, T47D cells were used as a positive control. Fig. 3. *top panel*, show the results of RT-PCR and the *next panel down* presents the relative SNCG mRNA levels after normalization with the signal of GAPDH. These results demonstrate that of 10 patient samples, 9 patients displayed SNCG expression in tumor samples. SNCG mRNA was not detected in five tumor-adjacent normal tissues (N1, N5, N7, N8, and N10), whereas low levels were detected in other four normal samples (N2, N3, N6, and N9), and a high level was found in N4. The detection of SNCG expression in the five tissue samples, supposed to be normal, prompted us to reexamine the original tissue slices; we found that all of the adjacent "normal" tissues contained regions of infiltrating tumor cells, which were the likely source of SNCG mRNA detected in these tissue samples.

Second, to determine whether the expression of SNCG in breast tumors is associated with hypomethylation, we selected five patient samples of both tumor and normal tissues for genomic bisulfite sequencing. The sequencing results are summarized in Table 3. The N/T pair number 7, which were SNCG-negative for both the normal tissue and the tumor, showed methylation in five of five clones for normal tissue and four of six clones for tumor. Importantly, the methylation occurred at the CpG sites 2, 5, 7, and 10–15, exactly matching the methylation pattern seen in cell lines. The N/T pair number 8, in which the normal tissue did not show any expression and the tumor showed low level of SNCG mRNA, the exon 1 was methylated in all of the clones from the normal tissue and was partially demethylated in the tumor tissue. In N/T pair number 6, the exon 1 was unmethylated for both normal and tumor tissues, which correlated well with the SNCG expression. In N/T pairs numbers 1 and 5, the exon 1 was unmethylated in tumors as well as in normal tissues, albeit with a lack of SNCG expression in normal tissues, suggesting that demethylation could occur before the gene expression. Taken together, the results from breast tumor tissues agreed to a large extent with the results from breast cancer cell lines and provided critical *in vivo* evidence to support the role of DNA methylation in the control of SNCG expression.

Table 2 *Characteristics of surgical breast tumor specimens*

The tumors were staged following standard American Joint Committee on Carcinoma/International Union against Carcinoma tumor-node-metastasis methodology. The SNCG mRNA expression was determined by RT-PCR and normalized with the mRNA levels of GAPDH as shown in Fig. 3. The double ++ sign indicates the ratio of SNCG mRNA:GAPDH mRNA > 1, the single + sign indicates a ratio of SNCG mRNA:GAPDH mRNA > 0.25, and the − sign indicates a ratio of SNCG mRNA:GAPDH mRNA < 0.2.

Tumor sample no.	Diagnosis	Histologic grade	Nuclear grade	Tumor cells positive axillary lymph nodes	ER	SNCG mRNA expression
T1	Infiltrating (95%) and *in-situ* carcinoma	III	na[a]	34/38	na	++
T2	*In-situ* and invasion duct carcinoma	na	na	11/15	na	++
T3	*In-situ* and infiltrating duct carcinoma	III	na	15/26	+	+
T4	*In-situ* and invasion duct carcinoma	III	III	0/22	−	+
T5	*In-situ* and invasion duct carcinoma	III	na	2/12	na	++
T6	*In-situ* and invasion duct carcinoma	III	III	7/18	+	++
T7	Invasive carcinoma, ductal type	III	III	0/13	na	−
T8	Invasive ductal carcinoma	II/III	II/III	na	−	−/+
T9	*In-situ* and invasive lobular carcinoma	II/III	II/III	7/16	+	+
T10	Invasive ductal carcinoma	III	III	5/18	na	+

[a] na, the information was not available; ER, estrogen receptor.

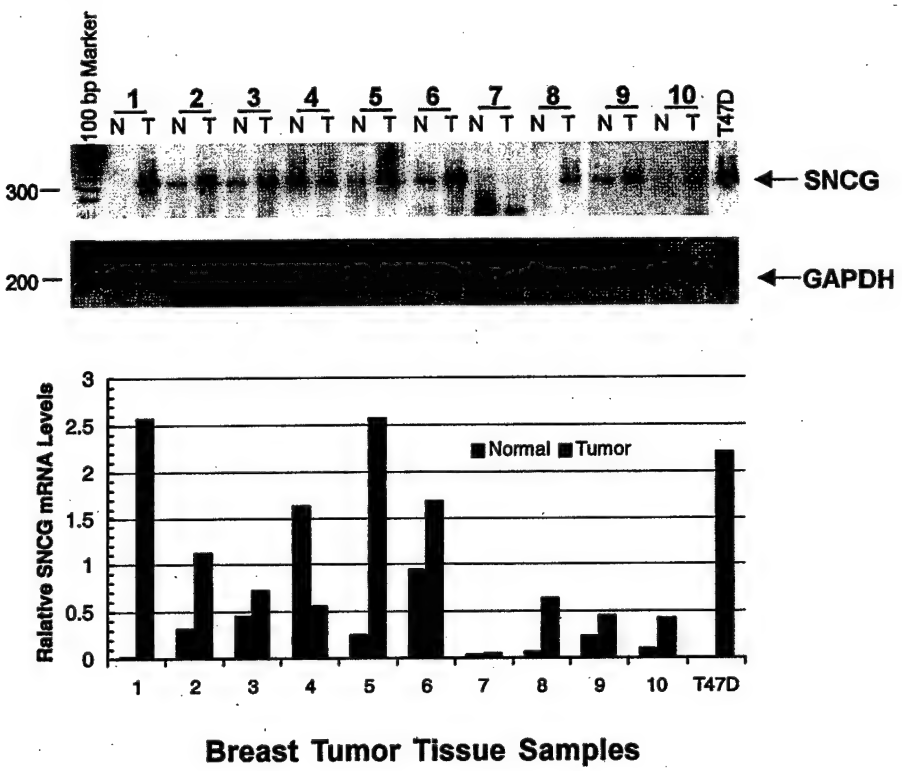

Fig. 3. Detection of SNCG mRNA and GAPDH mRNA in primary breast tumors and in matched normal breast tissue adjacent to tumors. RNA was isolated from 10 pairs of primary breast tissues; *T*, tumor tissue; *N*, normal breast adjacent to tumor. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide (*top panel*). The intensity of PCR product was scanned by Bio-Rad Fluro-S MultiImager system and quantified by the program of Quantity One. The relative SNCG mRNA levels are presented as the ratio of SNCG mRNA:GAPDH mRNA. SNCG-positive cell line T47D was used in this experiment as a positive control. The data shown are representative of two to three separate RT-PCRs.

Comparison of SNCG mRNA Expression and the Methylation Status of Exon 1 in Normal Breast Tissues with Invasive Breast Carcinomas. It is possible that SNCG mRNA detected in the tumor-adjacent normal tissues was produced by infiltrated tumor cells instead of normal cells. Alternatively, the genetic background of noncancerous tissue adjacent to the tumor cells has been changed, which could activate SNCG expression. We evaluated these possibilities by an examination of SNCG expression in 10 invasive tumors and in 6 normal breast tissues obtained from reduction mammoplasty of healthy individuals. RT-PCR analysis showed that of 10 tumor tissues, 8 displayed high expression of SNCG (Fig. 4*A*, *left panel*). In contrast, under the same conditions, RT-PCR did not detect SNCG expression in normal samples (Fig. 4*A*, *right panel*). The bisulfite genomic sequencing was done for five tumors and the results showed that all of the tumor samples contained mostly unmethylated *SNCG* gene, whereas the normal breast tissues contained methylated as well as unmethylated *SNCG* gene. The percentage methylation for normal breast and tumor samples is presented in Fig. 4*B*, and the difference in methylation was found to be statistically highly significant. ($P \leq 0.003$). Because the majority of genetic materials from normal breast tissue were derived from fat cells and fibroblasts, and only a small portion was actually from the epithelium, it is possible that the unmethylated allele belonged to other cell types.

We further extended our study to organoids. They were epithelial clumps obtained by digestion of normal breast tissue from one donor with collagenase and hyaluronidase at 37°C for 24–72 h. Fig. 5 showed the results of RT-PCR from the organoids before and after extensive cell culture to become finite HMECs. Whereas a very low level of SNCG mRNA was detected in the organoids in which epithelial cells were incubated in culture medium containing growth factors for only 24–72 h, a high level of SNCG mRNA was shown in the matching HMECs. Sequencing results showed that the organoids contained mostly methylated clones (75%) but the finite life span HMECs were completely unmethylated. These data combined with Fig. 1*C* suggest that SNCG is not expressed *in vivo* in normal mammary epithelial cells, however, during the initial selection and culturing *in vitro*, the *SNCG* gene expression is induced through demethylation by growth factors present in the culture medium. SNCG expression may stimulate these primary cultures of cells to proliferate.

To further study that SNCG expression, controlled by demethylation, contributes to the cell proliferation, a normal mammary epithelial cell line HMEC-184 was treated with cytokine OM, which has been previously shown to inhibit the proliferation of HMECs (31). Cells were treated with OM at a concentration of 50 ng/ml for the indicated intervals of time, and cell proliferation was determined by [^{3}H]thymidine incorporation assay. Fig. 6*A* shows that OM treatment led to 30% inhibition of cell growth after 1 day of treatment, whereas up to 60% of inhibition was observed after 3 days of treatment. RT-PCR analysis (Fig. 6*B*) showed that levels of SNCG expression in OM-treated cells was concomitantly reduced by 70% as compared with control. With bisulfite genomic sequencing, four of six clones were found to be methylated in OM-treated cells, whereas all five of the clones sequenced from the untreated cells were unmethylated.

Table 3 *Bisulfite sequencing of cloned PCR products from breast tumor specimens*

DNA was isolated from five pairs of primary breast tissues in which T represents tumor tissue and N represents normal breast tissue adjacent to tumor. Genomic bisulfite sequencing of the exon 1 region of SNCG was performed as described in the "Materials and Methods" section. Four to eight clones from each tissue sample were sequenced. The expression of SNCG mRNA was determined and described in Fig. 3.

Breast tissue samples	Total no. of clones sequenced	Exon 1 methylated	Exon 1 unmethylated	SNCG RT-PCR
N1	8	0	8	−
T1	8	1	7	++
N5	9	0	9	−
T5	7	1	6	++
N6	8	0	8	+
T6	8	1	7	+
N7	5	5	0	−
T7	6	4	2	−
N8	4	4	0	−
T8	8	6	2	−/+

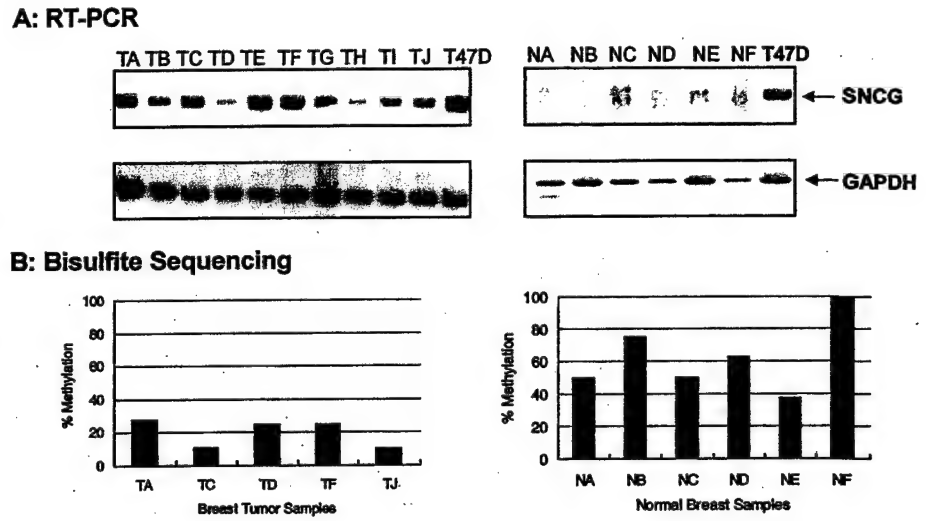

Fig. 4. RT-PCR analysis of SNCG mRNA expression in breast tissue samples from healthy individuals and from patients with advanced breast carcinomas. *A*, six normal breast tissues *NA–NF* (*right panel*) were obtained from healthy donors after mammoplasty, and RNA was isolated from 300–400 mg of tissue per sample. RNA form 10 tumor tissues of advanced breast carcinomas *TA–TJ* (*left panel*) was also isolated. RT-PCR to detect SNCG and GAPDH mRNA was conducted. RNA of T47D cells was used as a positive control. *B*, DNA was isolated from six normal breast tissues (*NA–NF*) and five tumors (*TA, TC, TD, TF, TJ*). Genomic bisulfite sequencing of the exon 1 region of SNCG was performed for 6–8 clones per sample as described in the "Materials and Methods" section. The *bar diagram*, the percentage of methylated clones. The difference in the degree of methylation between normal samples and tumor samples was evaluated using two-tailed Student's *t* test. Statistically significant difference ($P = 0.003$) between normal and tumor samples was reached.

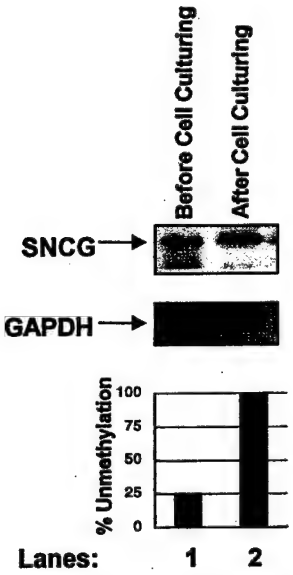

Fig. 5. Expression of SNCG in organoid before and after culturing. RNA and DNA were isolated from organoids (240L) obtained from normal breast tissue of one donor after mammoplasty (*Lane 1*) and the matching HMECs that were developed from the same organoids after continuous culturing (*Lane 2*). RT-PCR to detect SNCG and GAPDH mRNA was conducted as described earlier. The *bar diagram* (*bottom panel*), the percentage of unmethylated clones after genomic sulfite sequencing.

To directly examine the relationship between SNCG expression and cellular proliferation, SNCG was stably transfected into MCF-7 cells, and pool population was selected for both mock- and SNCG-transfected cells. Western blot detected an abundant amount of SNCG protein in MCF7-SNCG transfectants (Fig. 7*A*). The proliferation rate of MCF7-SNCG was compared with mock-transfected MCF-7 cells by using Cyquant cell proliferation assay kit. Fig. 7*B* shows that SNCG-transfected cells proliferate faster than mock-transfected cells, and a >2-fold increase in cell proliferation was observed after 5 days. Cell cycle analysis (Fig. 7*B*, *inset*) showed that SNCG-transfected cells have more cells in S phase (33.11 + 3.2 in SNCG-transfected cells compared with 22.4 + 2.3 in mock-transfected cells) and less in G_2-M (15.06 + 2.8) compared with mock-transfected cells (22.22 + 1.5). Overall, these data suggest that SNCG is a stimulating factor for cell proliferation; the SNCG expression is correlated with demethylation of the exon 1 and that the inhibition of cell growth leads to an increased DNA methylation at the CpG island of the *SNCG* gene, resulting in reduced expression.

Examination of SNCG mRNA Expression in Malignant and Normal Ovarian Epithelium-derived Cell Lines. To determine whether demethylation of exon 1 is also a causal factor for SNCG expression in ovarian cancer cells, the experiments of RT-PCR and *in vivo* genomic sequencing were conducted in five ovarian tumor-derived cell lines and five normal ovarian epithelium-derived cell lines (HIOs). The results of RT-PCR are presented in Fig. 8, *upper panel*, and the summarized sequencing data are shown in Fig. 9. Among the five cancer cell lines, OVCAR3 and OVCAR5 expressed high levels of SNCG mRNA, and these cells contained fully demethylated exon 1, whereas A2780 expressed a moderate level of SNCG mRNA, and exon 1 in these cells was partially methylated. In

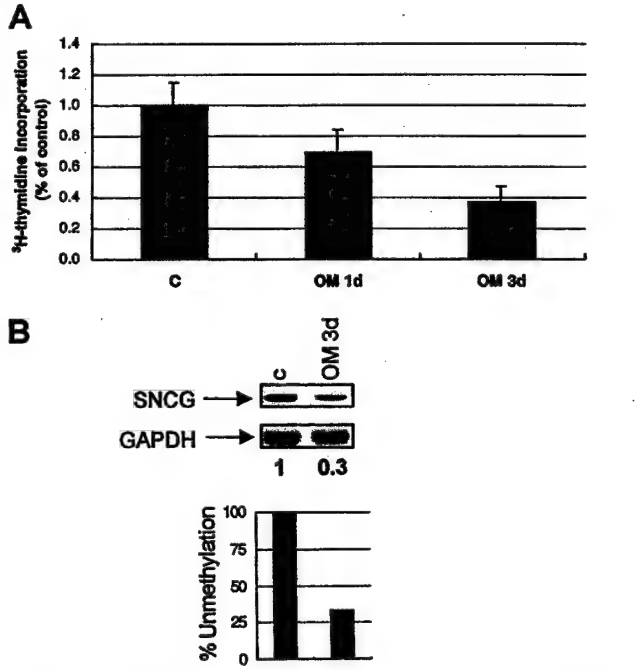

Fig. 6. Regulation of cell proliferation and SNCG mRNA expression and DNA methylation in HMEC-184 by OM. *A*, 1.2×10^4 cells were seeded in 24-well plate and treated with 50 ng/ml for 1 and 3 days. Cells were then pulsed with [^{3}H]thymidine for 16 h. The amount of radioactivity incorporated was determined by trichloracetic acid precipitation. *B*, mRNA expression and DNA methylation of SNCG in HMEC-184 by RT-PCR analysis and DNA bisulfite sequencing. Equal numbers of cells were seeded in 100-mm dishes for control and OM treatment of 3 days. The cells were then harvested for RNA and DNA isolation. The *bar diagram* (*bottom panel*), the percentage of unmethylated clones after genomic sulfite sequencing.

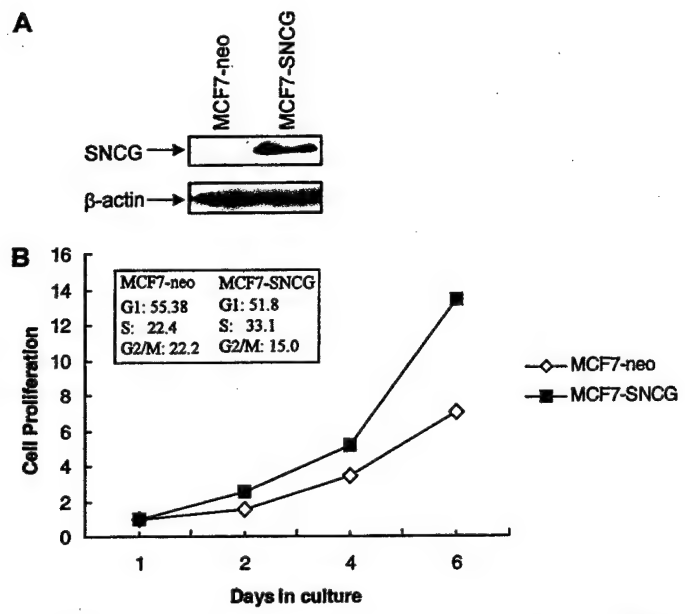

Fig. 7. Cell proliferation assay and cell cycle distribution of DNA in mock- and SNCG-transfected cells. *A*, MCF-7 was transfected with the pCIneo vector or with pCI-SNCG, and the pool populations were selected with 300 μg/ml G418. SNCG protein expression in the mock- and SNCG-transfected pool population of MCF-7 was examined by Western blot by using 20 μg of protein of total cell lysate. *B*, 1000 cells were seeded per well in black 96-well plates for each cell type and harvested at indicated intervals of time. The total DNA content was estimated using fluorescent dye as described in "Materials and Methods." The proliferation rate is expressed as the fold of DNA content in day 1 of each cell population. The data presented are derived from three separate experiments in which quadruplicate wells were used in each condition. The proliferation assay was also carried out in medium containing a different amount of FBS, and the proliferation rates of MCF7-SNCG cells were consistently higher than MCF7-neo cells in all of the assays. *Inset*, for cell cycle analysis, 2×10^5 cells were seeded in 100-mm dishes in RPMI containing 10% media. Next day, the cells were washed with PBS, and RPMI containing 0.5% of medium was added to the cells. After 48 h, the cells were harvested and analyzed by flow cytometry.

contrast, OVCAR4 and OVCAR8 did not express SNCG mRNA at all, and all of the 15 CpG sites of the CpG island in these two cell lines were completely methylated. Thus, SNCG expression appears to correlate very well with the demethylation of *SNCG* gene in ovarian cancer cell lines. Interestingly, the methylation pattern in ovarian cancer cells is different from that in breast cancer cells. The specific methylation patterns of ovarian cancer cell lines and breast cancer cell lines are presented in Table 4. Among the five normal HIO cell lines, SNCG mRNA was detected only in HIO-107 cells. Within eight clones of HIO-107 that were sequenced, four clones were completely methylated at every CpG site of CpG island, whereas the four other clones were nearly unmethylated. Other HIO lines showed no expression of SNCG mRNA, although all four of the cell lines displayed demethylation of the CpG island to a different extent. Thus, these data suggest that in normal ovarian epithelium-derived cell lines, besides DNA methylation, there are other factors that repress SNCG expression. Because all of the CpG sites in the CpG island of SNCG in ovarian cancer cells were methylated in SNCG-negative cells and unmethylated in SNCG-positive cells, it is possible to determine the methylation status by using a MSP assay. To explore this possibility, the bisulfite-modified genomic DNAs were amplified with the methylation-specific primers SNCG-M1F or SNCG-M2R, or the primers corresponding to unmethylated sequence, SNCG-U1CF and SNCG-U2R (Table 1). The *bottom panel* of Fig. 8 shows that a strong band of 102 bp (*SNCG-M*), corresponding to methylated sequence of exon 1 (−139 to −37), was amplified with the methylated primers from SNCG-negative OVCAR4 and OVCAR8 cells, but this band was not amplified from SNCG-positive OVCAR3 and OVCAR5 cells. In contrast to the methylated primers, the same region was specifically amplified using unmethylated primers from OVCAR3 and OVCAR5 but was not amplified from OVCAR4 and OVCAR8. MSP detected both methylated and unmethylated alleles from A2780. Thus, the MSP produced similar results as the direct sequencing. Likewise, the results of MSP of five HIO cell lines agreed to a large degree with the sequencing data and showed that HIO-107 and HIO-108 contained unmethylated and methylated alleles and HIO-103 and -105 contained mostly unmethylated alleles, whereas HIO-135 contained mainly methylated *SNCG* gene. These data suggest that MSP can reliably

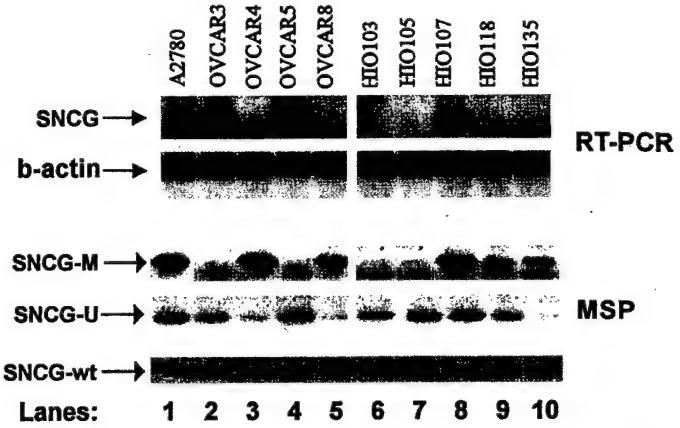

Fig. 8. Detection of SNCG mRNA in ovarian cancer and normal ovarian epithelium-derived (HIO) cell lines by RT-PCR analysis and the methylation status of exon 1 by MSP. *A*, the ovarian cancer and HIO cells were analyzed for SNCG mRNA and β-actin mRNA. *B*, MSP was used to assess the methylation status of SNCG CpG islands in each cell lines. Bisulfite-modified genomic DNA was used as template to amply exon 1 region −139 to −37. The PCR reactions with primers to detect methylated DNA and unmethylated DNA were performed separately. *SNCG-M*, methylated primers; *SNCG-U*, unmethylated primers; *SNCG-wt*, primers corresponding to the wild-type sequence of unmodified DNA The unmodified genomic DNA was amplified with primers corresponding to the wild-type sequence as a positive control for the quality of DNA.

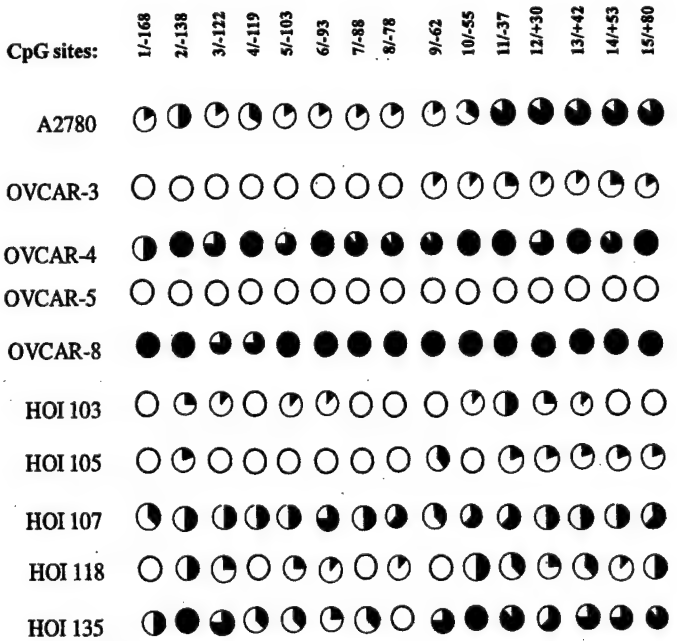

Fig. 9. Methylation status of CpG islands in SNCG exon 1 region in various ovarian cancer and HIO cell lines. CpG positions are indicated relative to the translation start codon, and *each circle* in the figure represents a single CpG site. For each cell line, the percentage methylation at a single CpG site is calculated from the sequencing results of 6–8 independent clones. ●, 100% methylation; ○, 0% methylation.

Table 4 *Comparison of methylation patterns of breast epithelium-derived cells with ovarian epithelium-derived cell lines*

	CpG position														
	1/−168	2/−138	3/−122	4/−119	5/−103	6/−93	7/−88	8/−78	9/−62	10/−55	11/−37	12/+30	13/+42	14/+53	15/+80
MDA-MB468	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	+	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	+	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	+	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	+	+	+	+	+	+	+
	0	100	0	0	100	0	100	0	66.7	100	100	100	100	100	100% methylation
MCF10A	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	−	+	−	−	+	−	+	−	−	+	+	+	+	+	+
	0	100	0	0	100	0	100	0	0	100	100	100	100	100	100% methylation
OVCAR-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+
	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	−	+	−	+	−	+	+	−	−	+	+	−	+	−	+
	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+
	50	100	75	100	75	100	87.5	87.5	87.5	100	87.5	87.5	100	87.5	100% methylation
OVCAR-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	100	100	75	87.5	100	100	100	100	100	100	100	100	100	100	100% methylation

distinguish the unmethylated *SNCG* from the methylated gene in ovarian cancer cells.

Reexpression of SNCG by 5-Aza-C Treatment. If the transcriptional silencing of SNCG can be attributed to the methylation of exon 1, then demethylating the gene with 5-Aza-C should lead to expression of SNCG in both breast cancer and ovarian cancer cells. To test this, we chose two SNCG-negative breast cancer cell lines (MDA-MB435 and MCF-7), two SNCG-negative ovarian cancer cell lines (OVCAR4 and OVCAR8), and one SNCG-negative HIO cell line (HIO-135). All of these cell lines contain methylated *SNCG* gene. Cells were exposed to different concentrations of 5-Aza-C for 4–6 days, and the medium and drug were replaced daily. Fig. 10*A* shows that, in breast cancer cells, SNCG mRNA expression was induced by low doses of 5-Aza-C (0.6 μM for MCF-7 and 1 μM for MDA-MB435), and its level was increased by higher concentrations of 5-Aza-C in a dose-dependent manner. In the two ovarian tumor lines, higher doses (10 μM) of 5-Aza-C were required to induce SNCG expression as compared with 1 μM for the HIO-135 cells (Fig. 10*B*). To confirm that reactivation of SNCG expression by 5-aza-C was the result of the demethylation of the exon 1, we isolated DNA from OVCAR4 and OVCAR8, which had been treated with a 10-μM concentration of 5-aza-C, and performed bisulfite sequencing. As expected, the CpG islands in these ovarian tumor cells became unmethylated (data not shown). In contrast to SNCG-negative cell lines, 5-aza-C treatment of OVCAR3 and OVCAR5 did not further increase the level of SNCG expression. Collectively, these data clearly demonstrate that reactivation of SNCG expression by 5-Aza-C was the direct effect of demethylation of exon 1 and was not a secondary effect caused by other factors the expression of which were changed by the treatment.

DISCUSSION

The onset of cancer is associated with the silencing of the tumor suppressor genes and activation of proto-oncogenes. Previous studies have suggested that SNCG could function as an oncogene in breast cancer cells. It has been demonstrated that exogenous expression of SNCG in breast cancer cells (MDA-MB435) led to a significant

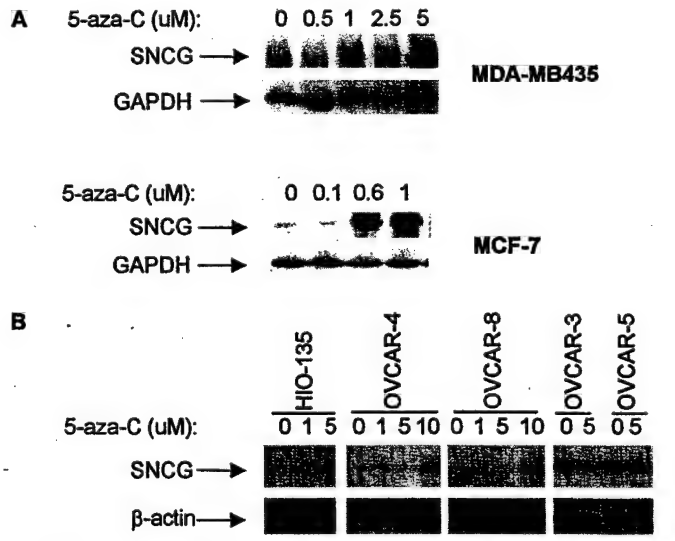

Fig. 10. Re-expression of SNCG mRNA after 5-Aza-dC treatment in breast and ovarian cancer cell lines. Breast cancer cell lines MCF-7 and MDA-MB 435 were treated with indicated doses of 5-Aza-dC for 4 days (*A*) and ovarian cancer cell lines and HIO 135 line were treated with 5-Aza-dC for 6 days (*B*). RNA from each cell line was isolated at the end of treatment, and the expressions of SNCG mRNA and GAPDH or β-actin mRNA were determined by RT-PCR.

increase in cell motility and invasiveness in cell culture and to a profound augmentation of metastasis in nude mice (24). Furthermore, exogenous expression of SNCG in MCF-7 cells significantly stimulated the growth of MCF-7 cells under anchorage-dependent (Fig. 7) and anchorage-independent conditions (33), whereas blocking SNCG expression with SNCG antisense mRNA markedly reduced the colony formation of T47D cells (25). Although previous studies by our group and others have shown that SNCG was abnormally expressed in advanced-stage breast carcinoma and ovarian carcinoma and that it is likely to be important in the pathogenesis of these neoplastic diseases, the mechanism(s) for its aberrant expression was unclear. In this report, we extensively examined the relationship between demethylation or hypomethylation of SNCG CpG island, and the expression of this candidate oncogene in breast cancer and ovarian cancer under both *in vitro* and *in vivo* conditions. From our present study, four important findings emerged.

First, in breast cancer cell lines, SNCG expression correlates with complete demethylation of the exon 1 region. By contrast, three of four SNCG-negative cell lines showed specific methylation at the CpG sites 2, 5, 7, and 10–15, suggesting that methylation at these sites is sufficient to block the expression of this gene in cell culture. More importantly, these same CpG sites were found methylated in primary breast tumor tissues and in MCF-10A, a cell line that originated from normal mammary epithelium. Thus, these data argue that, in normal mammary epithelium, SNCG is methylated at the specific CpG sites, resulting in blocking of transcription.

Semiquantitative RT-PCR, a highly sensitive detection method, failed to identify SNCG expression in six of six normal breast tissues from healthy women without cancer. This result provided new evidence to support original findings that SNCG expression was confined to malignant mammary epithelial cells. *In vivo* genomic sequencing, however, detected both methylated and unmethylated *SNCG* gene from the same normal breast tissue samples. We tentatively interpret this discrepancy thus: the unmethylated genes could be derived from other cell types such as fat cells, fibroblasts, or mononuclear leukocytes, and the lack of expression in these cells could be controlled by other mechanisms. However, our results cannot exclude the possibility that in normal mammary epithelium, SNCG is partially methylated and that partial methylation is sufficient to block its expression. Additional studies to examine SNCG expression and methylation in nonepithelial cell types of breast tissue will be needed to resolve this discrepancy. The unmethylated exon 1 in normal tissues adjacent to tumors suggests that demethylation may precede expression. A similar observation has been reported in benign and malignant colon neoplasms (9). Although the benign tumors do not express HGH, α and γ *globin* genes were hypomethylated in the promoter region of these genes just like malignant tissues, thereby suggesting that alteration in DNA methylation precedes malignancy.

The second important finding in this study is that DNA methylation also plays an important role in SNCG expression in ovarian cancer cells. Similar to breast cancer cells, an inverse relationship between exon 1 methylation and SNCG expression was found in various ovarian cancer and HIO cell lines. However, there are two characteristics that are unique and distinguish ovarian-derived epithelial cells from breast-derived epithelial cells. First, the 15 CpG sites in ovarian cancer cells were all methylated instead of being selectively methylated at the hot spots that were identified in breast cancer cells. Secondly, in ovarian cells, partial methylation permitted SNCG expression, albeit at a lower level, whereas SNCG expression could not be detected in breast cancer cells in which the exon 1 was partially and heterogeneously methylated. These differences suggest that, whereas partial methylation in the exon 1 is adequate to inhibit SNCG expression in breast cancer cells, complete methylation of the CpG island is required for silencing SNCG in ovarian cancer cells. The third finding of this study is that SNCG is expressed in three primary HMEC lines (184, 048R, and 240L) that have limited life span and that the expression correlates with hypomethylation of the exon 1. By contrast, SNCG is not expressed in normal mammary epithelial cells *in vivo* nor in the established cell line MCF-10A, and is detected at a very low level in organoids before extensive culturing. These observations suggest that SNCG expression and demethylation of the exon 1 are regulated possibly by growth factors that are present in culture medium during the establishment of HMEC cell lines. Consequently, *SNCG* gene product further stimulates cell proliferation of the primary mammary epithelial cells that normally have low growth potential. Although this is a hypothetical scenario, there is some evidence to support this hypothesis. Celis *et al.* (34) have reported that a group of genes that were not expressed in the original bladder transitional carcinomas became expressed when the tumor tissues were incubated in culture medium for a very short time (1–2 days). Synuclein was found within this group. Future studies to clearly define the function of this protein in neoplasm will provide insight to understand the molecular mechanisms that control the methylation status of *SNCG* gene.

The last important finding is that we provided direct evidence to demonstrate a stimulating role of SNCG in the growth of breast cancer cells. Transfection of SNCG into MCF-7 cells resulted in an increased proliferation rate of cells. Conversely, we also demonstrated a correlation between reduced growth rate and decreased SNCG expression. Importantly, we showed the coordinated changes in cell growth, SNCG mRNA level, and methylation status of the exon 1 of *SNCG* gene. When the HMECs, which express high levels of SNCG, are arrested by OM or by serum starvation (data not shown), the SNCG levels decrease, and the exon 1 becomes hypermethylated. At the present, the normal cellular functions of SNCG are largely unknown. Future studies to clearly define the function of this protein in neurons as well as in neoplasm will provide insight to understand the molecular mechanisms that control the methylation status of *SNCG* gene.

Currently, in the cancer research field, DNA hypermethylation has received considerable attention, and DNA hypomethylation is studied inadequately. In fact, the original observation of altered DNA methylation in cancer was hypomethylation (16). A recent study of genome-wide screening for normally methylated human CpG islands has found a considerable number of genes containing methylated CpG islands. We believe that our studies provide a clearly defined example supporting the hypothesis that abnormal hypomethylation contributes to cancer formation.

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Blockade of AP1 Transactivation Abrogates the Abnormal Expression of Breast Cancer-specific Gene 1 in Breast Cancer Cells*

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Breast cancer-specific gene 1 (*BCSG1*) is not expressed in normal breast tissue but is highly expressed in the vast majority of invasive and metastatic breast carcinomas. When over-expressed, BCSG1 significantly stimulates the proliferation and invasion of breast cancer cells. The accumulated evidence suggests that the aberrant expression of BCSG1 in breast carcinomas is caused by transcriptional activation of the *BCSG1* gene. However, the transcription factors that activate BCSG1 transcription have not been identified. In this study, we extensively investigated the role of AP1 in BCSG1 expression in breast cancer cells. We demonstrate that there are two closely located AP1 binding sites residing in the first intron of the *BCSG1* gene. Mutation of either AP1 motif on the BCSG1 promoter constructs markedly reduces the promoter activity. We further show that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increases BCSG1 mRNA expression and up-regulates BCSG1 promoter activity through the intronic AP1 sites. The effect of TPA on BCSG1 transcription is also demonstrated under *in vivo* conditions in intact cells by using chromatin immunoprecipitation assays that show the TPA-induced binding of c-Jun to the chromatin region encompassing the intronic AP1 sites. Finally, to examine the direct effect of AP1 transactivation on BCSG1 expression, we established stable cell lines of T47D that express the dominant negative mutant of c-Jun, TAM67. RT-PCR and Western blot analyses demonstrated that levels of BCSG1 mRNA and protein in TAM67 transfectants were drastically reduced as compared with mock-transfected cells. Furthermore, inhibition of BCSG1 expression by blocking AP1 transactivation produced a similar repressive effect on cell growth as that by expressing BCSG1 antisense mRNA. We show that the anchorage-independent growth of T47D cells expressing either TAM67 or BCSG1 antisense mRNA is significantly inhibited. Taken together, we provide strong evidence that AP1 plays an overriding role in the transcription of the *BCSG1* gene and that blockade of AP1 transactivation down-regulates BCSG1 expression and suppresses tumor phenotype.

Previous studies conducted by differential DNA sequencing and *in situ* hybridization have identified *BCSG1*[1] (1), also referred to as synuclein γ (2) or persyn (3), as a breast cancer-specific gene because of its distinct expression pattern in breast tissues. BCSG1 is not expressed in normal breast tissue or tissues with benign breast diseases but is highly expressed in the vast majority of stage III/IV breast carcinomas (1, 4). BCSG1 expression in advanced breast carcinomas is not merely adventitious but plays a positive role in the process of invasion and metastasis. It has been demonstrated that exogenous expression of BCSG1 in breast cancer cells leads to a significant increase in cell motility and cell proliferation in cell culture (5, 6), as well as a profound augmentation of metastasis in nude mice (5). Thus far, *BCSG1* gene mutations or amplifications have not been found by examination of breast carcinoma specimens and breast cancer cell lines. Therefore, it was suggested that the abundant expression of BCSG1 protein and mRNA in breast carcinomas is not caused by mutation or gene amplification but by transcriptional activation (3, 4).

To elucidate the molecular and cellular mechanisms that control BCSG1 transcription in breast cancer cells, we recently isolated a 2.2-kb fragment of human *BCSG1* gene that includes 1 kb of the 5′-flanking region, exon 1, and intron 1 (7). Sequence analysis indicates that the exon 1 region contains a CpG island. In *vivo* genomic bisulfite sequencing demonstrates that the BCSG1 CpG island is totally unmethylated in BCSG1-positive SKBR-3 and T47D cells but partially methylated in BCSG1-negative MCF-7 and HepG2 cells (7). This suggests that DNA demethylation may be an important factor contributing to the aberrant expression of BCSG1 in breast cancer cells. However, treatment of MCF-7 or HepG2 cells with a specific methyltransferase inhibitor 5-aza-2′-deoxycytidine that demethylated the CpG island of BCSG1 only induced low levels of BCSG1 mRNA in these cells, suggesting that there are DNA methylation-independent mechanisms responsible for the abundant expression of BCSG1 in T47D and SKBR3 cells. It is possible that demethylation of cytosine of the CpG dinucleotide motifs within exon 1 may allow initiation of gene transcription. Upon release of transcriptional silencing, BCSG1 transcription is further activated by transcription factors that interact with the cis-acting elements residing in the regulatory regions of the *BCSG1* gene.

To identify the regulatory sequences we analyzed the promoter activities of the 2.2-kb genomic fragment of *BCSG1* gene and its various deletion derivatives. Transient transfection

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[1] The abbreviations used are: *BCSG1*, breast cancer-specific gene 1; AP1, activator protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoresis mobility shift assay; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OM, oncostatin M; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT, reverse transcription; RSV-βgal, Rous sarcoma virus-β-galactosidase.

assays showed that the luciferase reporter construct containing the 5′-flanking region and the exon 1 of the *BCSG1* gene produced a low level of luciferase activity; this basal activity is mediated primarily through a GC-rich region located immediately upstream of the transcription initiation sites. However, the promoter reporter activity was markedly increased by inclusion of the intron 1 region of the *BCSG1* gene. Further deletion analysis localized a consensus AP1 binding site (TGACTCA) in the intron that was largely responsible for the increased promoter activity (7). These previous studies suggest that the activator protein AP1 may regulate BCSG1 transcription, possibly through the intronic AP1 binding site.

Because BCSG1 expression stimulates breast tumor disease progression and AP1 has been demonstrated to play an important role in tumorigenesis (8–10), activation of BCSG1 transcription might be one of the molecular mechanisms responsible for the promoting role of AP1 in breast cancer disease development and progression. Therefore, in this report, we extensively investigated the functional role of AP1 in BCSG1 transcription. Our studies clearly demonstrate that *BCSG1* is a new target gene of AP1 and that AP1 plays an overriding role in BCSG1 transcription in breast cancer cells.

MATERIALS AND METHODS

Cells and Reagents—Human breast cancer cell lines SKBR-3 and T47D were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO). Human hepatoma cell line HepG2 was cultured in Eagle's minimum essential medium supplemented with 10% FBS. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma. Antibodies directed to BCSG1 (E-20), c-Jun (sc-44), c-Fos (sc-52), and β-actin were obtained from Santa Cruz Biotechnology. The plasmid pcDNA3.1-TAM67, encoding a c-Jun mutant, TAM67, was generously provided by Dr. Michael J. Birrer, National Institutes of Health (Rockville, MD).

Transient Transfection Assays—SKBR-3 and T47D cells cultured in 24-well plates at a density of 0.12×10^6 cells/well were transiently transfected with a total of 200 ng of reporter DNA and 2 ng of pRL-SV40 (*Renilla*, Promega)/well mixed with Effectene reagent (Qiagen, Valencia, CA). 40 h after transfection, luciferase activities were measured using the Promega Dual Luciferase Assay System. HepG2 cells seeded in 24-well plates were transfected by the method of calcium phosphate coprecipitation (11). The BCSG1 promoter luciferase reporter constructs were cotransfected with RSV-βgal vector to normalize the transfection efficiency. For every cell line, triplicate wells were assayed for each transfection condition, and at least three independent transfection assays were performed for each reporter construct. For TPA treatment, the transfected cells were cultured in medium containing 0.5% FBS overnight then treated with TPA or Me_2SO as control for 24 h before harvesting cell lysate.

RT-PCR Analysis of BCSG1 mRNA—Cells were lysed in Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, Texas), and total cellular RNA was isolated according to the vendor's protocol. Assays of reverse transcription were conducted with random primers (Promega) using Superscript II (Invitrogen) and 2 μg of total RNA in a volume of 20 μl/reaction. The reaction mixture was incubated at 42 °C for 50 min and then was diluted to a total volume of 100 μl. PCR reactions using 2 μl of RT reaction product with a total of 38 cycles were performed for BCSG1 and 26 cycles for GAPDH. The PCR conditions were 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s with initial activation of the enzyme at 94 °C for 1 min.

The primers sequences were as follows: BCSG1 forward primer, 5′-CAAGAAGGGCTTCTCCATCGCCAAGG-3′; BCSG1 reverse primer, 5′-CCTCTTTCTCTTTGGATGCCACACCC-3′; GAPDH forward primer, 5′-CCATCACTGCCACCCAGAAGAC-3′; GAPDH reverse primer, 5′-GGCAGGTTTTTCTAGACGGCAG-3′.

Electrophoresis Mobility Shift Assays (EMSA)—Nuclear extracts were prepared as described previously (12). The 30-bp double-stranded oligonucleotide probe containing the BCSG1 intron 1 region (+596 to +624) (7) was annealed and end-labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Each binding reaction was composed of 10 mM HEPES, pH 7.8, 2 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 2 μg of poly(dI-dC), 1 μg of bovine serum albumin, and 4 μg of nuclear extract in a final volume of 20 μl. Nuclear extracts were incubated with 1 ng of ^{32}P-labeled probe ($40–80 \times 10^3$ cpm) for 10 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine, 1.5 mM EDTA, pH 8.5) at 180 V for 2–3 h at 4 °C. The gels were dried and visualized on a PhosphorImager. In competition analysis, nuclear extracts were incubated with a 100-fold molar excess of unlabeled competitor DNA for 5 min prior to the addition of the labeled probe. For supershift assays, antibody was incubated with nuclear extract for 30–60 min at room temperature prior to the addition of the probe.

The sense sequences of the EMSA probes were as follows: BCSG1-AP1, 5′-GGGGAGGTCAAGCCAATGACTCAGCTCTGG-3′; AP1-MU1, 5′-GGGGA*AT*TCAAGCCAATGACTCAGCTCTGG-3′; AP1-MU2, 5′-GGGGAGGTCAAGCCAAT*CCTA*CAGCTCTGG-3′; Sp1, 5′-TTCGAAAC-TCCTCCCCCTGCTAG-3′. The mutated sequences are italic and underlined.

Site-directed Mutagenesis—Mutant BCSG1 promoter reporters BCSG1967AP1-MU1 and AP1-MU2 were generated by site-directed mutagenesis on the template DNA (BCSG1967) with the QuikChange® site-directed mutagenesis kit (Stratagene, San Diego, CA). The correct mutation at the consensus AP1 site (TGACTCA → TcctaCA) in the vector BCSG1967AP1-MU2 and at the AP1-like sequence on the reverse strand (GAGGTCA → GAatTCA) in BCSG1967AP1-MU1 were verified by dideoxy sequencing.

Chromatin Immunoprecipitation (ChIP) Assays with Antibody to c-Jun—Control cells or TPA 24-h-treated cells were cross-linked with 0.37% formaldehyde at 37 °C for 10 min, total cell lysate was isolated, and the genomic DNA was sheared to between 400 and 600 bp by sonication. Chromatin immunoprecipitation with antibodies to c-Jun or normal IgG as negative control was performed using aliquots of lysate obtained from 5×10^6 cells as described previously (13). The immunocomplex was heated at 65 °C for 4 h to reverse the cross-linking between DNA and proteins. DNA was digested with 40 μg/ml proteinase K at 45 °C for 1 h and purified by repeated phenol/chloroform extraction and ethanol precipitation. The purified DNA (designated as bound) was dissolved in 20 μl of Tris buffer (10 mM Tris, pH 8.5). The DNA isolated using the same procedure with omission of the step of immunoprecipitation was designated as the input DNA and was diluted 100-fold prior to be used in the PCR reaction. The bound and input DNA were analyzed by PCR (30 cycles) with primers (AP1CHIP-5′, AP1CHIP-3′) that amplify BCSG1 intron 1 region (+549 to +748). The PCR conditions were 94 °C for 5 min, 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The 200-bp PCR product was visualized on 2% agarose gels stained with ethidium bromide.

To examine the mutational effect of intronic AP1 sites on endogenous c-Jun binding, SKBR-3 cells were transiently transfected with BCSG1 promoter luciferase construct BCSG1967AP1-MU2. The transfected cells were untreated or treated with TPA for 24 h. The ChIP assays were conducted as described above with two sets of primers that amplify the plasmid DNA and the endogenous *BCSG1* gene separately. To amplify the intronic AP1 sites from the plasmid DNA, the primers AP1CHIP-5′ and AP1CHIP-3′E were used yielding a 240-bp PCR product containing the BCSG1 sequence (+549 to +707) plus an 80-bp vector sequence. The intensities of the PCR products were scanned using a BioRad Fluoro-S MultiImager System and quantified by the program Quantity One. Different amounts of template DNA were tested in PCR reaction to ensure a linear range of DNA amplification.

The following primers were used in the ChIP assays: for endogenous AP1 sites, AP1CHIP-5′ (5′-agtgttccctccccgcatcctctcc-3′) and AP1C-HIP-3′ (5′-ggacaggacagaatgtgggggacaa-3′); for the mutated AP1 site on plasmid DNA, AP1CHIP-5′ (5′-agtgttccctcccccgcatcctctcc-3′) and AP1C-HIP 3′E (5′-CCGTACAGGCCTAGAAGTAAAGGC-3′).

Stable Transfection of T47D Cells with TAM67—TAM67 expression vector (pcDNA3.1-TAM67) and the empty vector (pcDNA3.1) were transfected separately into T47D cells using Effectene reagent. After 10–14 days, individual clones resistant to G418 at a concentration of 500 μg/ml were isolated and propagated into individual stable cell lines. Western blotting using anti-c-Jun antibody confirmed that the 29 kDa TAM67 was expressed only in the T47D-TAM67 cells and not in the vector-transfected cells.

Stable Expression of BCSG1 Antisense mRNA in T47D Cells—A 285-bp DNA fragment corresponding to the exon 1 region (−169 to +116) of *BCSG1* gene was amplified from the plasmid pBS-BCSG1759 (7) and subcloned into the *Eco*RI site of the expression vector pcDNA3.1(−). The antisense or sense orientation of exon 1 in the pcDNA3.1 vector was determined by restriction enzyme digestion and was verified by DNA sequencing. Vectors expressing BCSG1 antisense

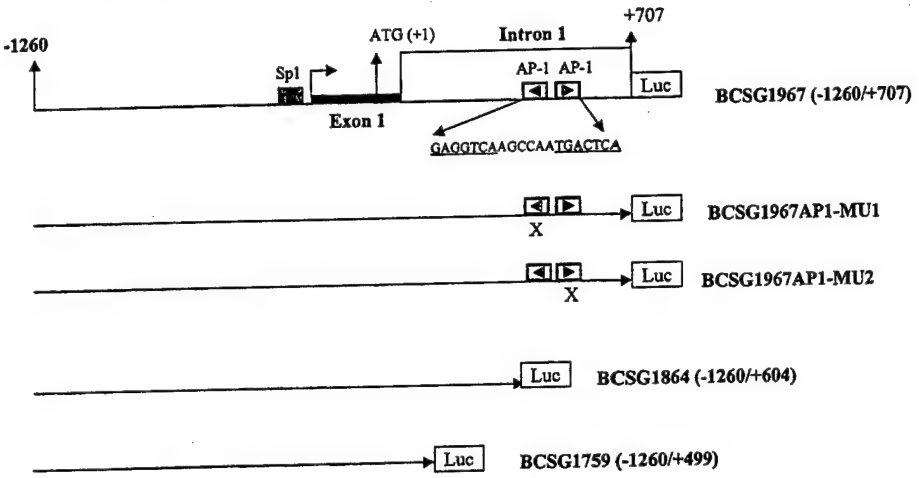

FIG. 1. **Diagram of BCSG1 promoter luciferase reporter constructs.** BCSG1 genomic fragments with 3′ truncations were cloned into pGL3-basic luciferase vector. The positions of the 5′-flanking region, exon 1, and intron 1 are numbered relative to the adenosine nucleotide of the ATG translation start codon. The ATG translation start codon at position +1 was mutated in all vectors to guarantee the correct translation of luciferase gene product.

mRNA (pcDNA-BCSG1-As) or BCSG1 sense mRNA (pcDNA-BCSG1-S) was separately transfected into T47D cells using Effectene reagent. Stable cell lines were established using the same procedures as described for TAM67 cell lines. The expression of BCSG1 antisense and sense mRNAs (285 bp) was confirmed by RT-PCR reaction. For antisense mRNA, the primer sets are: T7 as the forward primer, 5′-TAATACGACTCACTATAGGG-3′, and BCSG-Wf as the reverse primer, 5′-ACGCAGGGCTGGCTGGGCTCCA-3′. The primer sets for detection of sense mRNA are: T7 as the forward primer, 5′-TAATACGACTCACTATAGGG-3′ and BCSG-Wr as the reverse primer, 5′-CCTGCTTGGTCTTTTCCACC-3′.

Western Blot Analysis—Total cell lysates were isolated from cells as described previously (14). Fifty μg of protein of total cell lysate per sample was separated on 15% SDS-PAGE, transferred to nitrocellulose membranes, blotted with rabbit anti-BCSG1 polyclonal antibody (1:2000 dilution) or rabbit anti-c-Jun polyclonal antibody using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Membranes were stripped and reblotted with anti-β-actin monoclonal antibody to normalize the amount of protein loaded on gels.

Soft Agar Colony Assays—Anchorage-independent growth was carried out in 24-well culture plates. The bottom layer consisted of 375 μl of 5% FBS/RPMI containing 0.5% agar. The top layer consisted of 375 μl of 5% FBS/RPMI containing 0.33% agar and 2×10^4 cells. The cells were cultured in an atmosphere of 5% CO_2/95% air under saturating concentrations of humidity at 37 °C. After 7 days, the number of colonies was counted under a Nikon microscope at 200× amplification. Eight fields were randomly selected in each well using a Whipple glass ring with a 10 × 10 grid, and colonies of >10 cells and 6–10 cells were counted separately.

RESULTS

Our previous study identified a consensus AP1 binding site (TGACTCA) at the region +612 to +618, relative to the translation start codon, within the first intron of the *BCSG1* gene (7). An in-depth sequence analysis identified a second AP1-like sequence (TGACCTC) at positions +605 to +598 on the reverse strand of the intron 1. These two AP1 motifs are separated by only six nucleotides. To determine whether these two AP1 sequences are both functionally involved in BCSG1 transcription, mutations were introduced individually on each site to block AP1 binding. The BCSG1 luciferase reporter constructs carrying the wild-type and the mutated AP1 sites are depicted in Fig. 1. These reporters were transiently transfected into SKBR-3, T47D, and HepG2 cells. As shown in Fig. 2, the promoter activity of BCSG1967 was 5–7-fold higher than BCSG1759 and BCSG1864, which do not contain the intronic AP1 sites. Mutation at either AP1 site markedly reduced the promoter activity of BCSG1967 in all three cell lines. It appeared that disruption of the consensus AP1 site (AP1-MU2) produced a stronger inhibitory effect on BCSG1 transcription than the mutation at the AP1 homologous sequence (AP1-MU1). To confirm that the decreased promoter activities of the mutant vectors were caused by disruption of the binding of AP1 to the intronic AP1 sites, we conducted a competition binding assay using a ^{32}P-labeled oligonucleotide probe containing the AP1 motifs and nuclear extract of SKBR-3 cells (Fig. 3). EMSA detected one specific DNA protein complex that was supershifted by anti-c-Jun antibody but not by anti-c-Fos, suggesting that the AP1 DNA complex is composed mainly of a c-Jun homodimer. Formation of AP1 complex was not inhibited by a 100-fold molar amount of an oligonucleotide containing a Sp1 binding site but was completely inhibited by competition from a 100-fold molar amount of unlabeled AP1 probe (*lane 2*). The AP1-MU2 mutation totally lost the ability of the mutated oligonucleotide to compete for the binding, whereas the binding capacity of the oligonucleotide AP1-MU1 was decreased to 30% of the wild-type sequence, suggesting that the change of nucleotides on AP1-MU1 has a less damaging effect on AP1 binding than that of AP1-MU2. Taken together, these results demonstrate that AP1 motifs located on the sense strand and the antisense strand of intron 1 are both required for BCSG1 transcription.

We were interested in determining whether the intronic AP1 sites are involved not only in the basal transcription but also participate in the regulated transcription of BCSG1 by agents that induce AP1 transactivation. Because TPA is a well known AP1 activator (15, 16), we examined the effects of TPA on BCSG1 promoter activity. Treatment of cells with TPA for 24 h stimulated the BCSG1 promoter activity 3.1-fold in SKBR-3 cells, 2.3-fold in T47D, and 4.9-fold in HepG2 cells. Mutation of the AP1 site (AP1-MU2) completely abolished induction (Fig. 4*A*). EMSA showed that the elevated BCSG1 promoter activity was accompanied by increased AP1 DNA binding activity at the intronic AP1 sites in TPA-treated SKBR-3 and T47D cells (Fig. 4*B*). The stimulating effect of TPA on BCSG1 transcription was confirmed further by measuring the endogenous BCSG1 mRNA. RT-PCR analysis showed that TPA treatment increased BCSG1 mRNA 2.1-fold in SKBR-3 cells, whereas the mRNA level of *GAPDH* was not altered (Fig. 4*C*).

To demonstrate that the induction of BCSG1 transcription by TPA is the direct result of increased c-Jun binding to the intronic AP1 sites under *in vivo* conditions in intact cells, we performed the ChIP assays, which directly examined the binding of c-Jun to the chromatin region encompassing the BCSG1 intronic AP1 sites. Control and TPA-stimulated SKBR-3 cells were treated briefly with formaldehyde to cross-link DNA-binding proteins to chromatin. The isolated chromatin was subjected to sonication followed by immunoprecipitation with rabbit anti-c-Jun antibody or rabbit normal IgG as a negative control for nonspecific binding. DNA from the immunoprecipitate was isolated. From this DNA, a 200-bp fragment of the

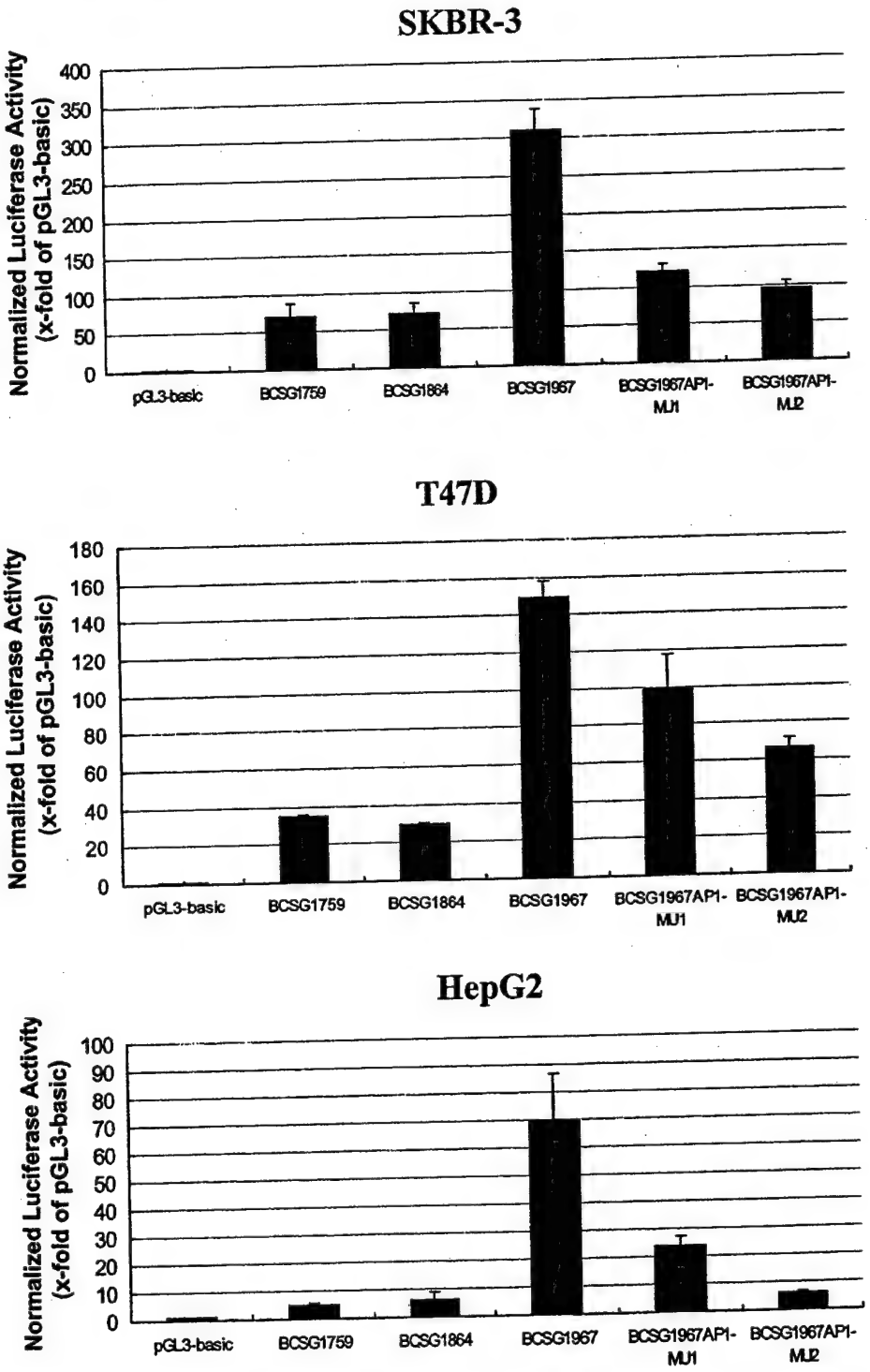

FIG. 2. **Analysis of luciferase activities of BCSG1 promoter constructs containing wild-type or mutated AP1 sites in the first intron.** BCSG1 promoter luciferase reporters were transiently transfected into SKBR-3 and T47D cells along with pRL-SV40 vector. 40 h after transfection, cells were harvested and dual luciferase activities were determined. In HepG2 cells, RSV-βgal was cotransfected with BCSG1 promoter vectors to normalize the transfection efficiency. The normalized luciferase activity is expressed as fold of control vector pGL3-basic, and the results represent means ± S.D. of triplicates.

BCSG1 intron 1 region surrounding the AP1 sites was amplified. Fig. 5*A* shows that the level of c-Jun cross-linked to the BCSG-AP1 sequence was increased ~5-fold in TPA-stimulated cells as compared with the control after normalization with the input DNA.

To further examine the effect of AP1 mutation on TPA-induced binding of c-Jun to this intron 1 region *in vivo*, SKBR-3 cells were transiently transfected with BCSG1 promoter luciferase construct BCSG1967AP1-MU2. The transfected cells were untreated or treated with TPA for 24 h. The ChIP assays were conducted using two sets of primers, which amplified the plasmid DNA and the endogenous DNA separately. To amplify the intronic AP1 sites from the plasmid DNA, the primers AP1CHIP-5′ and AP1CHIP-3′E were used, which yielded a 240-bp PCR product containing the BCSG1 sequence (+549 to +707) plus 80-bp vector sequence. As shown in Fig. 5*B*, whereas the binding of c-Jun to the endogenous BCSG1 intronic AP1 region was increased 3.6-fold by TPA, the binding of c-Jun to the transfected DNA containing the mutated AP1 site was not significantly increased in TPA-treated cells. The results of ChIP assays clearly confirmed our *in vitro* studies, demonstrating that the interaction of c-Jun with the intronic AP1 sites is important for the basal as well as TPA-induced transcription of the *BCSG1* gene in breast cancer cells.

To obtain direct functional evidence that AP1 controls BCSG1 transcription, we employed the approach of the dom-

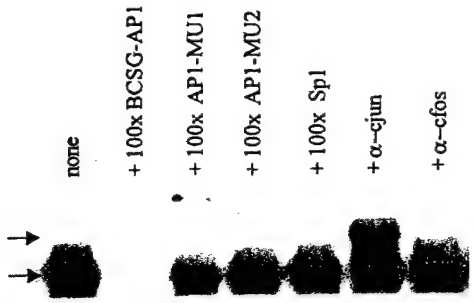

FIG. 3. **EMSA of nuclear proteins interacting with the intronic AP1 motifs.** A 30-bp double-stranded oligonucleotide, designated BCSG-Ap1, was radiolabeled and incubated with 4 μg of nuclear extract prepared from SKBR-3 cells in the absence (*lane 1*) or presence of 100-fold of molar amounts of unlabeled competitor DNA (*lanes 2–5*) or in the presence of antibodies to c-Jun (*lane 6*) or c-Fos (*lane 7*). The reaction mixtures were loaded onto a 6% polyacrylamide gel and run in TGE buffer at 180 V for 2.5 h at 4 °C. An EMSA using nuclear extract of T47D or other breast cancer cell lines showed similar results.

inant-negative mutant to inhibit the AP1 transcriptional activity. TAM67 is a deletion mutant of c-Jun that lacks the transactivation domain. TAM67 has been demonstrated to act as a potent inhibitor of AP1 transactivating activity by forming homodimers with full-length c-Jun and heterodimers with c-Fos and to quench the activity of AP1 complexes (17). After transfection of pcDNA-TAM67 vector into T47D cells and selection of resistance to neomycin G418, several stable clones were established along with the control clones that were transfected with the empty vector pcDNA3.1. Western blot analysis using an anti-c-Jun antibody shows that the 29-kDa c-Jun mutant was expressed only in TAM67 clones but not in T47D or the control clones (Fig. 6*A*). The inhibitory function of TAM67 on AP1 transactivation was examined by using an AP1 luciferase reporter, pAP1-Luc, which contains seven AP1 sites upstream of a basal promoter (Stratagene). Fig. 6*B* shows that compared with T47D and mock-transfected cells, the AP1 promoter activities in all three TAM67 clones were reduced to nearly the base-line level. These data clearly demonstrate that the AP1 transcriptional activity in T47D cells was greatly suppressed by expression of TAM67. Thus, RT-PCR assays were conducted to detect BCSG1 mRNA in TAM67 cells and in the control cells. Fig. 7*A* shows that a single band of 337 bp corresponding to the coding region of BCSG1 was readily detected in T47D cells as well as in the cells transfected with the empty vector. In contrast, this band was nearly undetectable in all clones that express TAM67, demonstrating that BCSG1 transcription in TAM67 cells was strongly inhibited. Western blot using specific anti-BCSG1 antibody shows that the amounts of BCSG1 protein expressed in TAM67 cells were decreased to levels below detection (Fig. 7*B*). Moreover, we compared BCSG1 promoter activity in TAM67 cells with that in mock-transfected cells. Fig. 7*C* shows that the promoter activity of BCSG1967 was markedly decreased in all three TAM67 clones as compared with the clones of empty vector, whereas the promoter activity of BCSG1864, which does not contain the intronic AP1 sites, was not significantly affected by expression of TAM67. Taken together, these results provide strong evidence that AP1 is a critical transcription factor for BCSG1 transcription through interaction with its recognition sequences residing in the first intron.

Previously we had shown that ectopic expression of BCSG1 in MCF-7 cells stimulated cell proliferation (6). To determine the effect of inhibition of BCSG1 expression by blocking AP1 transactivation on cell growth, the growth rates of TAM67 cells were compared with untransfected T47D and the mock-transfected cells in soft agar. Fig. 8 shows that after 7 days of

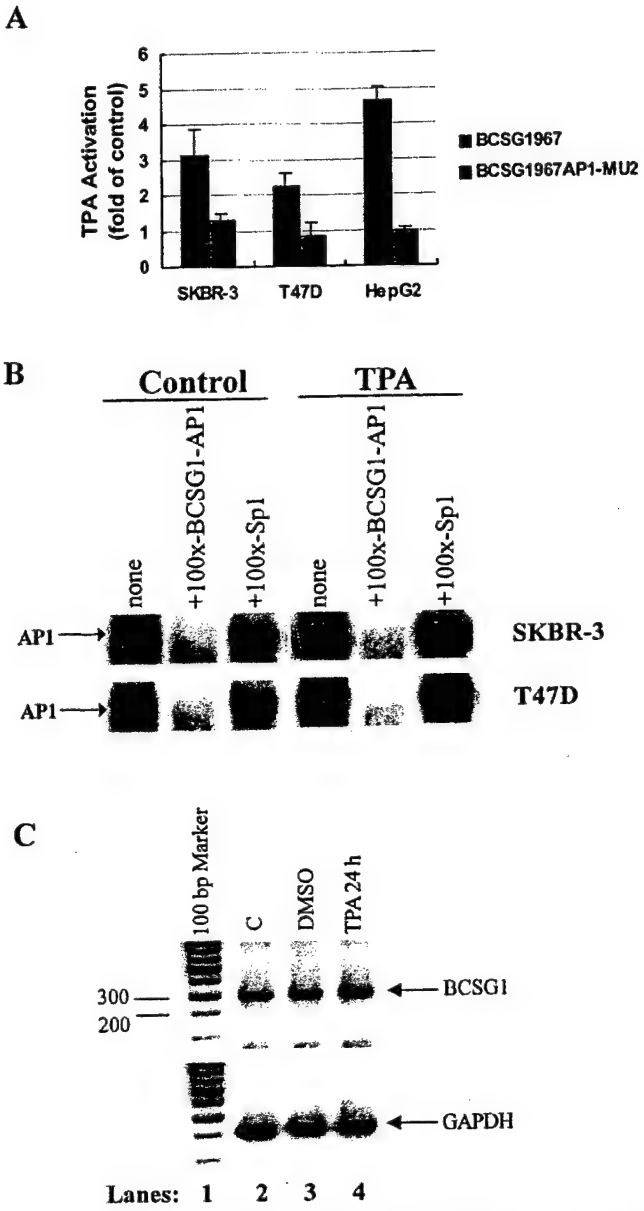

FIG. 4. **TPA stimulates BCSG1 transcription through the intronic AP1 sites.** *A*, promoter luciferase reporter assays in untreated control cells and in cells treated with 100 nM of TPA for 24 h. The normalized luciferase activities in TPA-treated cells are expressed as fold of control cells. *B*, EMSA of AP1 binding to the intronic AP1 sites with and without TPA treatment. Total cell lysates were harvested from cells treated with 100 nM TPA or with an equal volume of Me_2SO as control. Ten μg of protein was used in the EMSA. *C*, RT-PCR analysis of BCSG1 mRNA expression. Total RNA was harvested from untreated or TPA 24-h-treated cells or from cells treated with dimethyl sulfoxide (*DMSO*) alone. An RT-PCR reaction was performed to detect BCSG1 mRNA or GAPDH mRNA. RT-PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

culture in soft agar, the number and size of the colonies in cells expressing TAM67 were reduced to 10–20% of T47D cells.

The loss of BCSG1 expression in T47D-TAM67 cells may not solely account for the decreased cell growth in soft agar, as expression of TAM67 could inhibit other AP1-regulated genes that play roles in cell proliferation. To directly examine the effect of BCSG1 expression on the anchorage-independent growth of breast cancer cells, we constructed the vector pcDNA-BCSG1-As, which expresses the 5′ portion of the BCSG1 mRNA in an antisense orientation. As a negative control, the vector

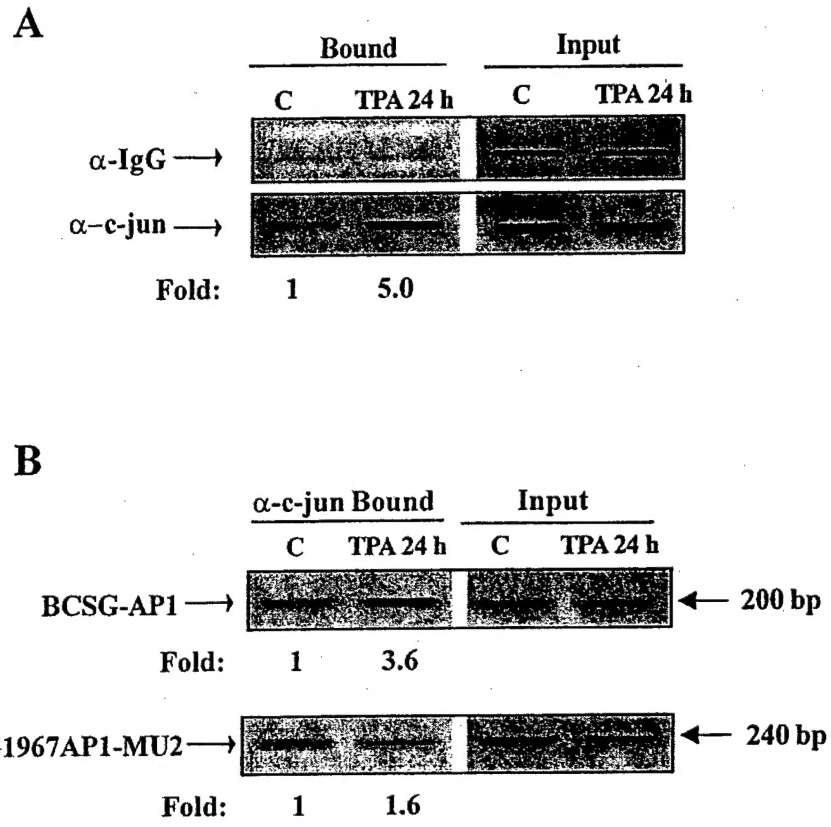

FIG. 5. **ChIP analysis for c-Jun association with BCSG1 intronic AP1 binding sites.** *A*, anti-c-Jun was used in a ChIP analysis followed by PCR to amplify a 200-bp region surrounding the intronic AP1 sites from genomic DNA isolated from SKBR-3 cells with or without TPA treatment. A rabbit normal IgG was used in the assay to assess nonspecific interactions. The PCR product was separately on 2% agarose gel, stained with ethidium bromide, and quantified using a BioRad Fluro-S MultiImager system. *Bound* represents the DNA co-immunoprecipitated with antibody, and *Input* represents the starting material before immunoprecipitation. The normalized level of c-Jun bound to the AP1 region in TPA-treated cells is expressed as fold of control cells. *B*, SKBR-3 cells were transfected with vector BCSG1967-MU2, and thereafter TPA was added to the medium for 24 h. The ChIP assays were conducted with primers that amplified the endogenous BCSG1 intron 1 region (*200 bp*) as described in *A* and with primers that amplified the plasmid DNA surrounding the mutated intronic AP1 site (*240 bp*).

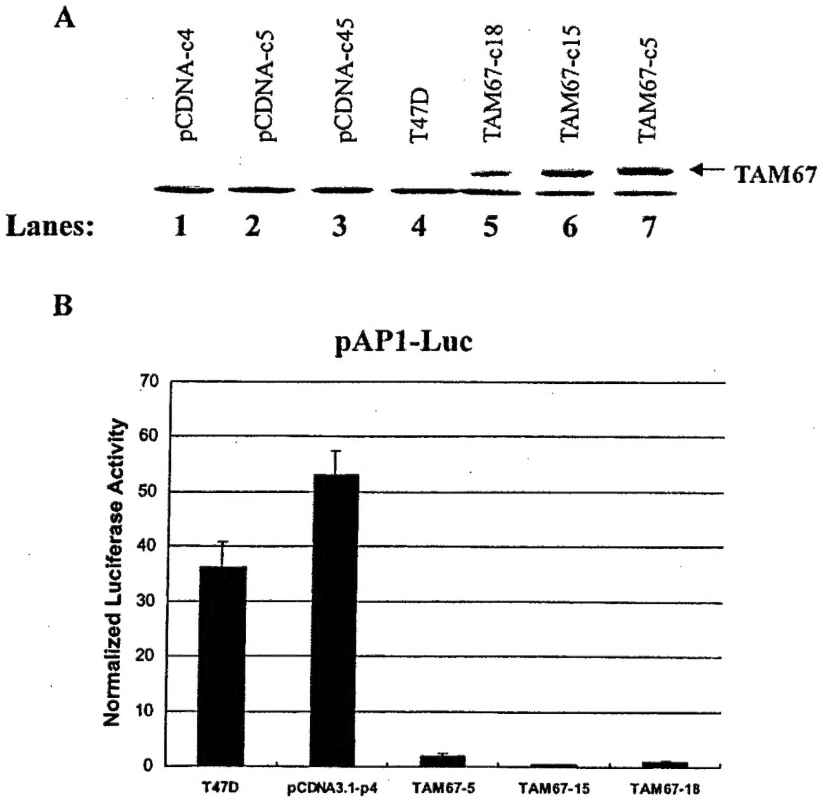

FIG. 6. **Expression of c-Jun mutant TAM67 in T47D cells.** *A*, Western blot analysis to detect TAM67 expression in stable clones. Total cell lysates were harvested from T47D- and mock-transfected clones and from pcDNA-TAM67-transfected clones. 50 μg of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis, and the TAM67 protein expression was examined by Western blot analysis using a rabbit polyclonal antibody specific to c-Jun. A band at a molecular mass of 29 kDa, corresponding to the deletion mutant of c-Jun, was detected in TAM67 clones but not in T47D- or mock-transfected clones. A band with a molecular mass of 15 kDa, detected by this antibody in all of the samples, was nonspecific. *B*, analysis of luciferase activity of an AP1 reporter construct pAP1-luc in cells with or without expression of TAM67. The vector pAP1-Luc was co-transfected with pRL-SV40 vector.

pcDNA-BCSG1-S was also made, which expresses the same mRNA sequence in a sense orientation. The plasmids pcDNABCSG-As and pcDNABCSG-S were transfected separately into T47D cells, and stable cloned were selected. Western blot analysis showed that the BCSG1 protein expression in the clones expressing BCSG1 antisense mRNA was significantly reduced to the levels of 25–40% of T47D (Fig. 9*A*), indicating that BCSG1 translation was subverted by the antisense mRNA. Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing BCSG1 antisense mRNA was significantly suppressed to a similar extent as in TAM67 cells (Fig. 9*B*), whereas the growth rate of the clone expressing

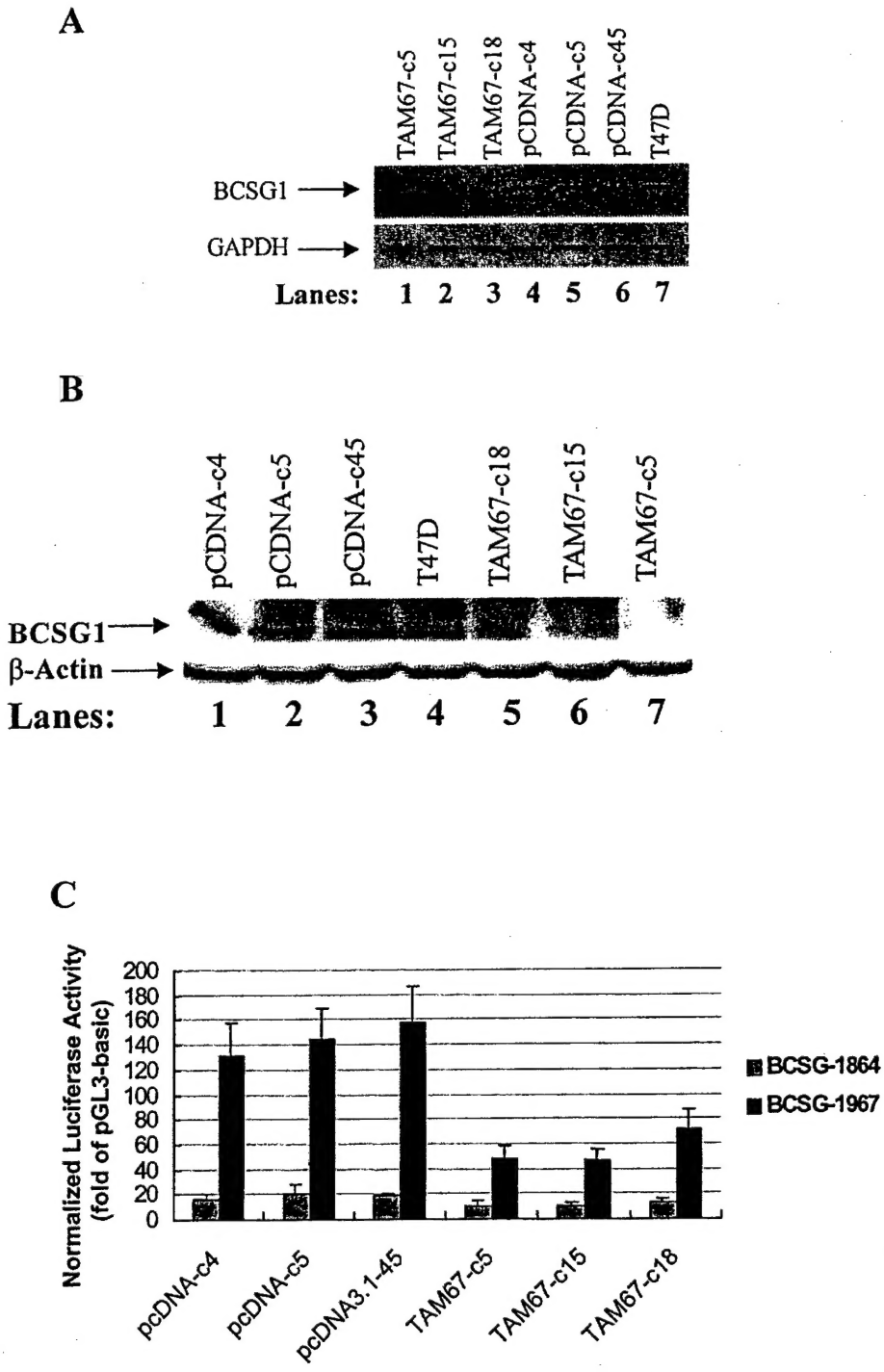

FIG. 7. **Expression of TAM67 inhibits BCSG1 expression.** *A*, RT-PCR analysis to detect BCSG1 mRNA and GAPDH mRNA expression. *B*, Western blot analysis to detect BCSG1 protein expression. 50 μg of protein from each sample was separately by SDS-polyacrylamide gel electrophoresis, and the BCSG1 protein expression was examined by Western blot analysis using a rabbit polyclonal antibody specific to BCSG1. *C*, BCSG1 promoter analysis. BCSG1 promoter luciferase reporter BCSG1967 and BCSG1864 were transiently transfected into mock clones or TAM67 clones along with pRL-SV40 vector. 40 h after transfection, cells were harvested and dual luciferase activities were determined.

BCSG1 sense mRNA was not statistically different from that in untransfected T47D cells.

DISCUSSION

Activator protein AP1 is mainly composed of c-Jun homodimers or c-Jun/c-Fos heterodimers. The AP1 complex mediates the transcriptional activation of a variety of genes through its specific binding to the DNA sequence TGACTCA, otherwise known as an AP1 site. It has been demonstrated that AP1 transcriptional activity is increased with the induction of the transformed phenotype and with neoplastic progression (8–10, 18, 19). In this study, we have demonstrated that AP1 has an overriding role in the control of BCSG1 transcription in breast cancer cells through specific interaction with two closely located AP1 sites residing in the first intron.

Although transcription of most of the mammalian genes is controlled by regulatory elements and enhancer sequences located in the 5′-flanking region, there are a number of genes that contain regulatory sequences in the first intron. For example, an active AP1 site present in the first intron of the p67 (*phox*) gene is critically important for its myeloid-specific expression (20). The lineage-specific expression of *MRP14* gene is controlled by a potent enhancer element located in the first intron as well (21). The 5′ promoter region of BCSG1 contains two AP1-like sequences at regions −1194 to −1188 and −488 to −482 relative to the translation start site. However, deletion

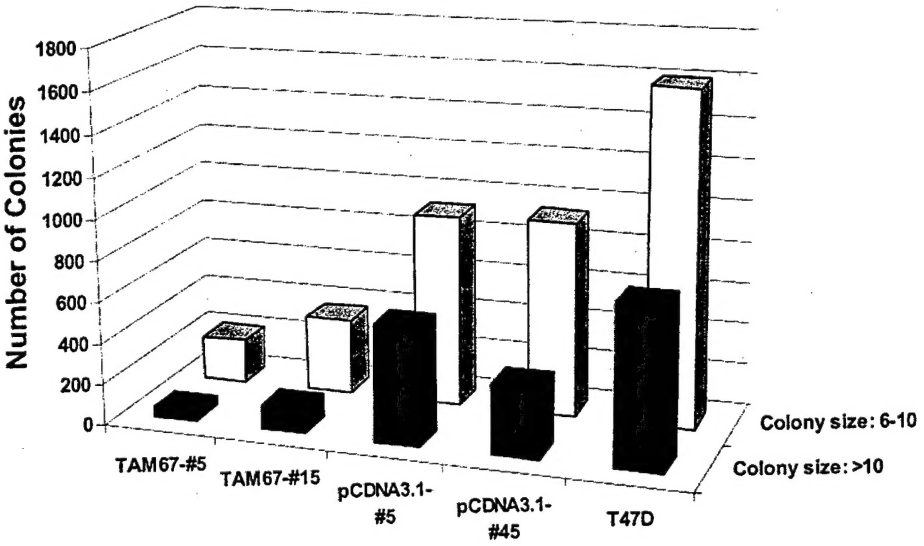

FIG. 8. **Comparison of the growth rates of T47D-TAM67 and T47D-pcDNA cells in soft agar colony assays.** Cells were plated in a 24-well plate in medium containing 5% FBS and 0.33% agar. The number of colonies at sizes large than 10 cells and between 6 and 10 cells was counted separately after 7 days.

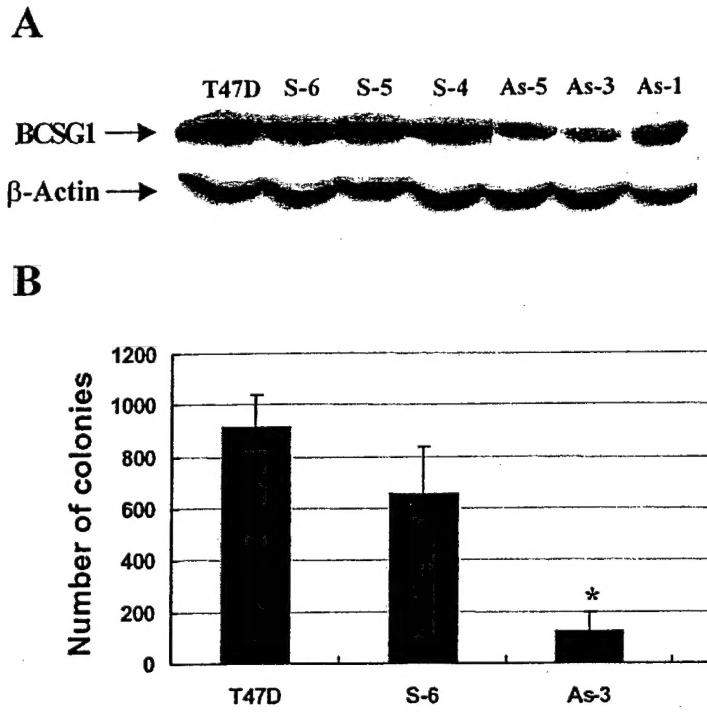

FIG. 9. **BCSG1 antisense mRNA blocked BCSG1 protein expression and suppressed the anchorage-independent growth of breast cancer cells.** *A*, Western blot analysis using 50 μg of protein from each sample was performed to determine the BCSG1 protein levels in T47D stable clones that express BCSG1 antisense mRNA or sense mRNA. The membrane was stripped and probed with anti-β-actin antibody to normalize the variation in protein loading. *B*, the growth rates of T47D cells and stable clones of T47D-BCSG1-As and T47D-BCSG1-S were compared by conducting soft agar colony assays as described in the legend for Fig. 8 except that the colonies containing more than 10 cells were counted and colonies smaller than 10 cells were excluded. A statistically significant difference ($p < 0.05$) between T47D and stable clones is indicated by an *asterisk*.

of these sequences did not affect the basal promoter activity of BCSG1, suggesting that they are not functionally involved in BCSG1 transcription (7). In contrast, the two intronic AP1 motifs appear to be critically important in BCSG1 transcription, as deletion or mutation to eliminate AP1 binding to these sites markedly reduced the basal- and TPA-induced promoter activities.

The direct effect of AP1 transactivation on BCSG1 transcription is demonstrated in T47D cells that express a dominant negative mutant of c-Jun, TAM67. Our supershifted EMSAs as well as ChIP assays show that c-Jun is the main protein component in the AP1 complex that binds to the intronic AP1 sites. Therefore, blocking AP1 transcriptional activity by expression of TAM67 should produce an inhibitory effect on BCSG1 transcription. We compared the levels of BCSG1 mRNA and protein in three stable clones of TAM67 with untransfected T47D and three mock-transfected clones. We show that expressions of BCSG1 in three mock-transfected clones are similar to the parental T47D cells, whereas BCSG1 mRNA and protein expressions in TAM67 clones were almost undetectable, demonstrating that blockade of AP1 transcriptional activity has a profound negative impact on BCSG1 transcription.

Previous studies in other cell types with TAM67 expression have shown that blocking AP1 transactivation inhibits the anchorage-independent growth of tumor cells and also neoplastic transformation (8, 18). In this study, by conducting soft agar colony assays we demonstrated that the growth rates of TAM67 cells are much slower than mock-transfected and untransfected parental T47D cells. TAM67-mediated inhibition of the anchorage-independent growth of T47D cells occurred, at least in part, because of the decreased expression of BCSG1, and we showed that inhibition of BCSG1 expression by producing BCSG1 antisense mRNA led to a strong growth inhibition of T47D cells in soft agar as well. These results provide additional evidence to support the hypothesis that BCSG1 might be a proto-oncogene, the expression of which stimulates the growth and transformation of breast cancer cells (2, 3, 5, 6).

Many extracellular stimulus affect cellular functions

through activation of AP1 transcriptional activity. AP1 motifs have been shown to be a converging site for several signal transduction pathways including ERK/MAP kinase, protein kinase C, and c-Jun N-terminal kinase pathways (22–24). In this study, we used TPA as a tool to explore the regulation of BCSG1 expression in breast cancer cells and we demonstrate that BCSG1 transcription was activated by TPA. Using both *in vitro* DNA binding assays and *in vivo* ChIP assays, we demonstrate that c-Jun, the major protein component of the AP1 DNA binding activity, interacts with the intronic AP1 sites, and this interaction is further stimulated by TPA. Previously, we have shown that BCSG1 transcription in breast cancer cell line H3922 was strongly inhibited by the cytokine oncostatin M (OM). It is tentatively to speculate that OM may affect BCSG1 transcription through the intronic AP1 sites.

The abnormal transcription of BCSG1 in breast cancer cells is likely controlled by multiple mechanisms. The results presented herein clearly demonstrate that AP1 is a key positive regulator for BCSG1 transcription in cells such as T47D or SKBR-3 in which the *BCSG1* gene is unmethylated. We have found that in MCF-7 or HepG2 cells, where *BCSG1* gene is methylated, activation of AP1 transactivation only increased the BCSG1 promoter reporter activity without a positive effect on the endogenous gene (data not shown). The transcription initiation sites of *BCSG1* gene are embedded in the CpG island; methylation in this region inhibits the interaction of basal transcriptional machinery with the promoter DNA and prevents the initiation of transcription. Thus, it is very likely that demethylation of the CpG island occurs in breast cancer cells as the first event, which allows BCSG1 to be transcribed. The abundant level of AP1 activity in tumor cells pushes the transcription of BCSG1 to a higher level.

BCSG1 expression is strongly associated with breast cancer disease progression and metastasis. When over-expressed, BCSG1 stimulates the proliferation and invasion of breast cancer cells. Our finding that AP1 is a key activator for BCSG1 transcription provides insightful information for further investigations to identify the different signaling pathways that lead to the activation of the *BCSG1* gene. Elucidation of the molecular mechanisms underlying BCSG1 transcription is important for the development of therapeutic approaches to block BCSG1 expression and to reverse the malignant phenotypes.

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